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EXPERIMENTAL PRODUCTION OF CONGENITAL MALFORMATIONS IN STRAINS OF INBRED MICE BY MATERNAL TREATMENT WITH HYPERVITAMINOSIS A

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A variety of methods is now available for producing congenital malformations in mammals by metabolic procedures.[1] Among these is a method employing relatively large doses of vitamin A, first devised by Cohlan,[2,3] who found with rats that it very successfully induced malformations such as exencephaly, hydrocephalus, spina bifida, cleft palate, cleft lip, brachygnathia, shortening of the maxilla, and gross eye defects. The efficacy of this technique was confirmed by Giroud and Martinet,[4] who in general found similar malformations but who were also able to demonstrate a differential effect on fetal development by starting treatment at several different periods of gestation.[5] Giroud and Martinet also paid special attention to Cohlan's observation[3] that the amniotic sacs of the abnormal embryos were swollen with excess, often blood-stained fluid. They found[6] that the amniotic sacs of fetuses with anencephaly contained more than 10 times the amount of fluid than was contained in sacs of fetuses without anencephaly. In addition, Giroud and Martinet used this means to produce exencephaly in order to investigate the morphogenesis of anencephaly,[7] and also made special studies of the malformations of the face[8] and of the urinary tract[9] induced by this method. Deuschle, Geiger and Warkany studied hypervitaminosis A-induced skeletal anomalies of the face leading to protrusion of the eye.[10]

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That similar malformations can be induced in mice by this technique was shown by Giroud and Martinet,[11] using Swiss Webster mice. Since it has been found in other experiments [12,13] that teratogenic agents varied in their effects according to the genetic constitution of inbred mouse strains employed, it seemed of interest to examine the effects of hypervitaminosis A upon the young of such inbred strains. In the course of these experiments, it was found that the malformations produced were in some respects similar to those observed in rats. However, malformations also made their appearance that had never been observed in other teratologic experiments.[14–16] Since many of these malformations simulated congenital malformations known to occur in human beings, it seemed worth while to report briefly upon the production of such developmental defects in experimental animals.

Material and Methods

Female mice of the A/J, DBA/1J, and C_3H/J inbred strains were placed with males of their own strains, one pair to a cage, and observed each morning thereafter for a copulation plug. The presence of a plug was taken to indicate that $\frac{1}{3}$ day had passed since conception. The impregnated female was then placed in a cage by herself. At $7\frac{1}{3}$, $8\frac{1}{3}$, $9\frac{1}{3}$, $10\frac{1}{3}$, $11\frac{1}{3}$, or $12\frac{1}{3}$ days after conception the females were given a single dose of 10,000 I.U. of vitamin A by mouth, by means of a blunt-ended 19-gauge needle and tuberculin syringe. The vitamin, originally containing 300,000 I.U. of vitamin A palmitate per cc. in sesame oil, was diluted to 50,000 I.U. per cc. with sesame oil, and 0.2 cc. was administered. The mice received Purina Laboratory Chow and fresh tap water *ad libitum* throughout pregnancy. The females were killed at $17\frac{1}{3}$ days after conception; that is, about 2 days before term. Resorption sites were counted, and the fetuses were removed, examined macroscopically, and then fixed in Bouin's solution for serial sectioning or in 95 per cent alcohol in preparation for staining of the skeleton by the Schulze-Dawson method.[17]

Results

To the time of writing, no strain differences in type or frequency of induced congenital malformations have been detected, and therefore the treated females and their offspring will be discussed without distinction as to strain.

A total of 92 females have so far been treated in the manner described. Treatment of 7 females at $7\frac{1}{3}$ days after conception caused all but one of the 53 young conceived to be resorbed. The animals treated after this time had at sacrifice 375 live young and 213 resorption sites. Malformations produced in these offspring affected many organ systems and were usually not compatible with postnatal life.

A widespread and complex syndrome of malformations resulted from a single administration of vitamin A to 34 females at $8\frac{1}{3}$ days after conception; these had 99 young and 139 resorption sites. Only a part of this syndrome was induced by treatment at $9\frac{1}{3}$ days after conception.

Eighteen females were treated at this time, and they had 97 young and 42 resorption sites. The defects that these two groups of offspring had in common will be considered first, and for this purpose these groups will be combined. Both groups had defects of the head, face and mouth, but those treated at 9⅓ days after conception had more severe abnormalities of these parts than those treated earlier.

Of the 196 young in these two groups, 193 were abnormal in one way or another (98.5 per cent). The most frequent defect was cleft palate, which occurred in 109 of the 154 young (70.8 per cent) whose mouths were examined (Fig. 1). Inspection of many palates was made impossible by the presence of an unusual anomaly, microstomia, which occurred in 64 cases (32.6 per cent; Figs. 2 to 4). This abnormality was characterized by reduction in size of the oral aperture, ranging from a moderate decrease to apparent obliteration of the opening. The defect was associated with anomalies of the skin, face, mouth, teeth, the underlying soft tissues and the cartilaginous and osseous skeletons. Included in this array were massive maxillomandibular ankylosis; reduction in length of the mandible due to foreshortening and absent or defective rami; heterotopic cartilage; lateral ankyloglossia (Fig. 26); absent, fused, supernumerary (Fig. 27), and ectopic teeth; absent masseter muscle; absent parotid, sublingual, and submaxillary glands and ducts; ectopic submaxillary glands, etc. These malformations and defects were recently described in detail.[16]

Another frequent defect of the head was exophthalmos of various degrees (Figs. 3 and 8), often with absence of the eyelids (32.2 per cent). The basis for this anomaly was great reduction in the size of the orbit, probably due to defective development of the facial skeleton, especially of the zygomatic arch. The eye itself, however, was normal. In addition, abnormalities of the pinna occurred quite often (57.7 per cent) and included external ears that were absent or small, defective, and placed in a low position (Figs. 2 to 4). Atresia of the eustachian tubes was also found in sectioned tissues.

Of great interest was an anomaly found in sectioned offspring from mothers treated at either of the two periods in pregnancy. This was an accessory thymus and various degrees of undescended thymus. In the latter, the organs ranged in location from those in a slightly elevated position, just below the thyroid, to those high in the neck, where a parapharyngeal or para- and moderately intra-pharyngeal location was occupied (Fig. 28). In those with this extreme form, remnants of the embryonic pharyngeal clefts (*ductus pharyngobranchialis III*) persisted, producing passages from the pharynx into the thymic tissue. In such animals the area of the normal position of the thymus in the

superior mediastinum just cephalic to the parietal pericardium appeared empty, and consisted of a space of about the size to accommodate the normal thymus (Fig. 5). Figure 6 shows the location and size of the thymuses in a control. The accessory thymus(es) occupied various positions in the neck, but in several cases a nodule of accessory thymus tissue was located in one or both eustachian tubes about half way along their courses (Fig. 25). The histologic structure of the ectopic thymus was denser than in controls of the same age, but was nevertheless recognizable. In specimens with these abnormalities there were no obvious defects of the parathyroid or thyroid glands except absence of the isthmus of the thyroid in some cases.

The only malformations other than those listed above produced by single treatment at 8⅓ or 9⅓ days after conception were of the tail. The defects of the tail (Fig. 8) were bent or wavy tail (2 and 27.8 per cent), short or abnormal tail (18.2 and 22.7 per cent), and absent tail (12.1 and 3.1 per cent).

In addition to the defects mentioned, treatment at 8⅓ days after conception also produced many anomalies that did not appear or appeared only rarely following treatment at 9⅓ days. Many of these anomalies were internal and were seen only in sectioned tissue: viz., 10 animals selected from the apparently severely deformed young, which were serially sectioned at 20 μ and stained with hematoxylin and eosin. These were defects of the central nervous system, median cleft mandible (10.1 per cent; Fig. 9), fused ribs (Fig. 7), cardiovascular anomalies, urogenital defects, anal atresia (15.1 per cent; Fig. 8), and others.

Externally visible malformations of the central nervous system were exencephaly and spina bifida aperta at the lower lumbar or sacral levels (Figs. 10 to 13). In two instances these defects appeared together in the same animal (Fig. 14). In the hypervitaminosis A experiments with rats, exencephaly occurred quite frequently,[3,4] but in the present study with mice only 5 examples of this defect were encountered. In sections of exencephalic young it was found that the cerebral hemispheres enclosed the lateral ventricles; the diencephalon, however, was not closed as is usual, but protruded between and overlay the hemispheres so that parts normally forming the roof of the third ventricle were contiguous with the skin of the temporal area. This is a common feature of exencephaly, for which the pathogenesis and structure have been described.[7] Although spina bifida aperta did not occur very often either (10 cases), 3 instances of spina bifida occulta were found in sections of 5 young with no outwardly overt defect of the spine and in whom this malformation was not suspected. It is possible, therefore, that spina bifida occulta occurred with quite appreciable frequency in these

animals. The structure of the spina bifida aperta was not greatly different from that previously described,[18,19] but the spina bifida occulta presented some novel features (Figs. 15 and 16).

One other defect of the central nervous system seen in sectioned material can probably best be called "crowded brain." In this defect the brain appeared too large for the cranium, with decrease or obliteration of the basal, pericerebellar, and perimedullary cisternas and reduction in size or complete closure of the rostral region of the aqueduct. Despite the latter phenomenon, no sign of hydrocephalus was noted. It is likely, however, that had the young been left *in utero* longer, hydrocephalus would have developed.

Five of the 10 sectioned animals exhibited crowded brain and a closed or narrow Sylvian aqueduct; all of these had spina bifida (4 aperta, 1 occulta). On the other hand, another 3 animals had brains with normal surrounding spaces; of these, 2 had normal spinal cords and 1 had spina bifida occulta. In the latter specimen with normal brain and spina bifida occulta, however, the aqueduct was narrow. The final 2 of the sectioned specimens had exencephaly, 1 with spina bifida aperta and the other with spina bifida occulta.

Another defect found was a craniopharyngeal duct. This occurred in 6 of the sectioned specimens, but was not present in sections of 6 controls of the same age or in sections of 4 young from mothers treated at 9⅓ days after conception. This anomaly consisted of a narrow passage from the rear part of the nasopharynx through the center of the presphenoid and ending at the base or in the parenchyma of the pituitary.

Malformations of interest were also found in the heart and great vessels. These included narrow and angulated ductus arteriosus (Figs. 17 and 18), complete transposition of the great vessels, entrance of the aorta and pulmonary trunk into a large right ventricle, high interventricular septal defect alone or combined with "overriding" aorta (Figs. 19 and 20), and a probable coronary aneurysm occupying a large space between the ventricles and communicating at its base with the right ventricle. The latter defect occurred in the specimen showing complete transposition.

Severe and complex urogenital malformations were present in 9 of the 10 sectioned animals. All 9 (6 males, 2 females, and 1?) had rectovesical fistulas (Fig. 29) and anal atresia. Renal abnormalities included bilateral renal agenesis; crossed ectopic kidney, the left kidney being shifted toward or to the right and fused to the right kidney (Figs. 21 and 22); midline fused and "lump" kidney (Figs. 23 and 24); and hydronephrosis. In the animal with renal agenesis, the ureters and genital ducts were also absent. Other defects of the latter structures

were double ureter, single midline ureter, ureteric atresia and absent ureter; confluence of the ureter with the genital duct and of the genital ducts with each other; anastomosis of the genital ducts and of the ureters; hydroureter; crossing of a genital duct from one side to the other and entering the bladder on the side opposite from which it originated; and ectopic point of entrance of the ureter and genital duct into the bladder. Further, there occurred vesical diverticula and ectopic ovaries.

In all 10 sectioned specimens the umbilical artery was absent. Mice of the fetal age examined normally possess a single umbilical artery, passing usually to the right of the bladder. In the defective animals, apparently in compensation for the lack of the umbilical artery, the superior mesenteric artery was greatly enlarged, traversed the abdominal cavity and entered the umbilicus. To such a degree had the superior mesenteric artery undertaken a leading role that the celiac artery originated from it instead of, as normally, from the aorta. In fact, caudal to the emergence of the superior mesenteric artery from the aorta, the latter was greatly reduced in caliber, being transformed into a relatively small vessel. In this condition it passed dorsal to the fused kidneys where it was greatly compressed against the inferior vena cava. The latter also traveled dorsal to the kidneys until it bifurcated more anteriorly than normal into the iliac arteries. In one instance the reduced aorta split into iliac arteries just cephalic to the midline "lump" kidney, one iliac descending dorsal and the other ventral to the kidney. The fetal venous system was usually normal.

Treatment at 10⅓, 11⅓ or 12⅓ days after conception produced syndromes of congenital malformations that were greatly alike and almost completely different from those resulting from the earlier treatments. The disorders following later treatment, however, were most severe and frequent when vitamin A was administered at 11⅓ days after conception. Thirty-three females treated at these times had 179 young and 48 resorbed implantation sites at sacrifice. A total of 106 of the 179 young were malformed in one way or another (59.2 per cent). Cleft palate without cleft lip occurred in 62 of 170 young examined (36.5 per cent). This was usually accompanied by foreshortened mandible.

Most characteristic of the defects produced by treatment at these periods were two types of limb abnormalities. The first consisted of various grades of micromelia, in the most severe instances of which the forearms and forelegs were completely absent. In the second type the second digit on the hands or feet was either very short, bent toward and syndactylous with the pollex or hallux, or it was completely absent, producing oligodactyly.

In cleared specimens there were various skeletal anomalies: absent femur, tibia, and fibula; moderately to severely short, thick and bent humerus, radius, ulna, femur, tibia, fibula, and ilium; absent deltoid process; and absent or partially absent sacral tuberculum of the ilium. In addition, various scapular and costal defects and anomalies of the base of the skull were noted.

No other defects were noted in sections of 2 offspring from females treated at 11⅓ days after conception.

Discussion

It is clear that hypervitaminosis A is a potent teratogen in mice, as it is in rats. The external defects occurred often, and their frequencies were easily determined. But it is not possible to state the percentage of internal defects, since all the specimens were not dissected or sectioned. Among the malformations are some that have occurred in previous experimental teratologic studies and have been repeatedly described. Such a defect is exencephaly. The form of this anomaly as produced by the administration of a large dose of vitamin A is well known; its gross aspects, indicating defects in closure of the neural tube, are similar to those produced in rats and mice by other teratogens.[20] Hypervitaminosis A-induced spina bifida aperta conformed, in general, to that previously described, in having an open neural plate, fused median spinal ganglia, overgrowth of nervous tissue, vertebral defects, etc.[18,19] The association noted in past studies, clinical and experimental, between spina bifida and the Arnold-Chiari malformation was also observed in the present investigation. In the rat, however, where the Arnold-Chiari malformation is simulated to a great extent following maternal treatment with trypan blue,[18,19] one characteristic of the malformation, the crowding of the brain, appears to be restricted to the metencephalon and myelencephalon. In the vitamin A-treated mice, however, the crowding was also present much further rostrally, usually starting at the optic chiasm. As in rats, the mice showed no herniation of the cerebellum through the foramen magnum, a cardinal sign of the Arnold-Chiari malformation in human subjects.

In selected sectioned young, malformations of great interest were found that have not yet been described as being produced experimentally; some of these occasionally occur in man. These are supernumerary upper and lower incisors, ankyloglossia lateralis, cervical thymus, coronary aneurysm, crossed ectopic kidney, rectovesical fistula, and absent umbilical artery.

Cervical thymus in man has been described quite recently by Michelson and Sender.[21] They recorded radiologic evidence of absence of the

organ in the chest of a child from whose neck was removed a hyperplastic thymus. In the older literature, absence of the thymus from the chest was also recorded by Harington[22] and Clark[23] both of whom apparently failed, however, to search the neck for primordia. In this century, cervical thymus was also described by Gruber[24] and Gilmour[25]; the latter and Weller[26] recorded the occurrence of less extreme variations of thymic maldevelopment. It is of interest that in a case reported by Gruber[27] there was also underdevelopment of the mandible and tongue, resulting in micrognathia, microglossia, and microstomia, as in specimens in the present study.

The only occurrence that we have discovered in human subjects of a situation resembling the intra-eustachian tube thymus described in this paper was that mentioned by Hagens.[28] This was a newborn, in whom there was a unilateral defect of the auditory apparatus with a mass of thymus tissue in the cavum tympani on the affected side.

In human beings, fusion of the tip of the tongue to the floor of the mouth or to the vault of the palate has been noted a number of times,[29–34] and has been termed superior ankyloglossia. However, lateral ankyloglossia is apparently a very rare anomaly in man, since we have been able to discover a record of lateral margin fusion of the tongue to the gum in one instance only.[35]

The latest case of coronary aneurysm described in man[36] brings to approximately 68 the number recorded in the literature, the latest surveys being those of Crocker, Sobin and Thomas,[37] Currarino, Silverman and Landing[38] and Scott.[39] The coronary aneurysm produced in the present experiment communicated with the right ventricle, as it frequently does in human beings.[40] In this instance, however, it occurred in a heart with transposition of the great vessels, and the arteriovenous fistula that would ordinarily have resulted was thus avoided.

All 10 specimens sectioned showed urologic maldevelopment; in 9 of these, severe disturbances were present, and in the tenth the left kidney lay in a moderately mediad position. The renal anomalies consisted mainly of deviations in location and in fusions. The kidneys were not reduced in size, and in some instances may have been enlarged. The one exception was the animal with bilateral renal agenesis. The ectopies and fusions occurred with a range of variation similar in some degree to that noted in human malformations. In man, one form, crossed renal ectopia with fusion, was the subject of a comprehensive review by Wilmer 20 years ago,[41] and the literature on this has only recently been brought up to date.[42]

Absence of an umbilical artery in human beings has been the subject of recent articles by Little[43] and by Benirschke and Bourne.[44] Little

examined 1,200 and Benirschke and Bourne 1,500 consecutive placentas, and found 16 and 15, respectively, with one missing umbilical artery, an incidence of about 1 in 100. This is a much higher rate of occurrence than is generally believed. Among these 31 cases, 10 had associated malformations in the infant. It is of interest that one child[44] had renal agenesis and atresia ani. This was observed in some of the mice in the present study. In a previous investigation[45] of absent umbilical artery, 27 instances were associated with defective infants, many of whom had renal defects and imperforate anus. In the studies cited,[43,44] roughly one third of placentas with an absent umbilical artery were associated with malformed infants; the authors, therefore, drew attention to the possible diagnostic significance of this vascular anomaly to the obstetrician.

Many experiments in teratogenesis have been made using mice as subjects. A partial list of the agents and the means used to disturb embryonic and fetal development of mice, culled from a recent review,[1] includes: deficiency of riboflavin, folic acid, and niacin; fasting, insulin, cortisone, ACTH and other pituitary preparations; radioiodine, estrogen, trypan blue, hypoxia, nitrogen mustard, and nicotine. It has been made clear from these investigations that quite different malformations and syndromes can be produced by different teratogens, so that even if treatment is administered at roughly the same times during pregnancy, many of these agents induce more or less distinct effects on development. Some examples of these effects in mice are: skeletal anomalies of long bones, esophageal atresia, and hydrocephalus, especially of the fourth ventricle, induced by riboflavin deficiency[13]; exencephaly, eye defects, cleft lip, cleft palate, median facial cleft, fused ribs, digital abnormalities, and gastroschisis, caused by folic acid deficiency[46–48]; and cleft palate and defects of the appendicular and axial skeletons, produced by niacin deficiency.[49] Fasting caused exencephaly, cleft palate, and costal and vertebral malformations.[46,50] Cortisone, compound F, and ACTH caused cleft palate only.[51–54]

An aspect of experimental teratology that has been greatly neglected in published reports is the extent and form of variability in the effects of teratogens on prenatal development. It is a common experience, we are sure, for great differences in severity and frequency of congenital defects to be found from litter to litter as well as from offspring to offspring within a single litter.

Two examples of the variability encountered in mice in our laboratory follow: In summarizing experiments carried out over a 5-year period, using cortisone administration in pregnant mice of a certain genetic constitution, it was discovered that the offspring of females treated between November and April showed cleft palate in 56 per cent; in those

treated from May to October this lesion appeared in 36 per cent.[55] This was a quantitative variation, apparently associated with season. In the second example the variation was qualitative. This experience occurred soon after the experiment forming the basis of this paper was brought to a temporary halt in early March, 1959. It was noticed, when the study was resumed several weeks later, that the same dose of vitamin A administered in the same manner at 8⅓ days after conception to mice of the same inbred strains no longer produced certain malformations that had been observed during the winter months. Malformations of the pinna and cleft palate continued to appear frequently, and mild and moderate microstomia was common. However, none of the defects of the central nervous system, anus, and tail that could be detected upon external examination in the young of females treated during the winter months were found in the litters from females treated in late March and April. In other words, of the entire array of anomalies produced in the first series, only a portion could be evoked in the second. While the basis for most of this variability is unclear, it need not remain entirely obscure. Such a problem was investigated and partly resolved in a series of experiments using cortisone.[56,57]

In contradistinction to variability of environmental origin, some of the variation found in certain studies appears to be of genetic nature. To detect such variability, it is necessary to utilize genetically uniform stocks of animals which are genetically distinct from all examples of other stocks. This condition of homogeneity can be observed only by the use of long-inbred strains. At the present time the only inbred strains of animals easily obtainable are strains of mice; some 200 now exist.[58] Such inbred strains are valuable for several reasons. The basis for this value is the uniformity and temporal constancy of their hereditary constitutions. Most important is the fact that, environmental conditions being equal, genetically determined responses of an inbred strain to an experimental manipulation will usually be less variable than the responses of a heterogeneous population. Consequently, one can repeat experiments and compare results with greater confidence of validity with inbred strains than with noninbred animals. Finally, because different strains may react quite differently to the same experimental procedure,[12,13,59,60] it is possible by use of inbred strains to investigate the genetic bases for these responses.

As stated previously, some of the effects of hypervitaminosis A in the prenatal development of mice are similar to those described in rats, i.e., exencephaly, exophthalmia, cleft palate, spina bifida, etc.[3,4,10] On the other hand, many of the anomalies induced in mice have not yet been reported in rats after such treatment. These are ankyloglossia,

dental defects, craniopharyngeal duct, cervical thymus, cardiovascular defects, ectopic fused kidney, rectovesical fistula, anal atresia, etc. To what extent these discrepancies are due to species differences and to what extent they have other explanations remains to be determined.

Summary

Congenital malformations were induced in the offspring of pregnant mice of inbred strains by the administration of relatively large amounts of vitamin A during the early stages of embryonic development. Anomalies produced affected the skin, ears, eyes, face, mouth, teeth, tongue, palate, thymus, ribs, brain, spinal cord, heart, great vessels, fetal circulation, kidney, ureter, bladder, genital ducts, rectum, anus, tail, and limbs. Some of the defects produced, not previously recorded in experimental teratogenesis, were supernumerary upper and lower incisors, ankyloglossia lateralis, craniopharyngeal duct, cervical thymus, coronary aneurysm, crossed ectopic kidney, rectovesical fistula, and absent umbilical artery. The occurrence of several of these defects in man is discussed.

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We are indebted to Hoffman-LaRoche, Inc., Nutley, N.J., for generous supplies of vitamin A.

LEGENDS FOR FIGURES

Except where indicated, photomicrographs were prepared from sections stained with hematoxylin and eosin.

FIG. 1. Ventral view of the head with the lower jaw removed, showing a cleft palate. × 5.

FIG. 2. Head of a young mouse with microstomia. Note the small oral aperture. × 6.

FIG. 3. Head of a mouse with "astomia." Note the apparent absence of an oral aperture and the exophthalmos. × 5.

FIG. 4. Offspring with microstomia and abnormal pinna. × 3.5.

FIG. 5. Section through the upper thorax, showing the space (arrow) where the thymus should be. Comparison with control in Figure 6 reveals displacement of the trachea relative to the position of the esophagus. × 11.

FIG. 6. Section through the upper thorax of a control mouse, showing the normal location and structure of the thymus (arrows). × 14.

FIG. 7. Ribs in an alizarin-stained specimen, showing extensive bilateral fusions. × 6.5.

FIG. 8. Young mouse with an absent tail and imperforate anus. There are also microstomia and exophthalmos. × 2.5.

FIG. 9. Median cleft mandible. × 6.

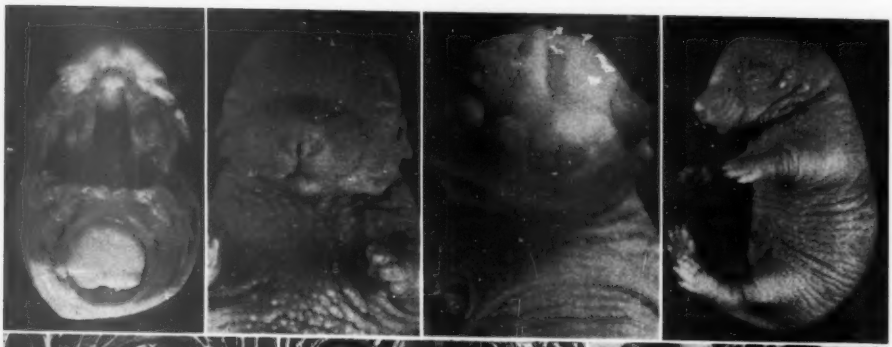
FIG. 10. Side view of a mouse, showing exophthalmos and exencephaly and the absence of the pinna and tail. × 2.5.

FIG. 11. Offspring with an open eye, exencephaly, gastroschisis, short lower trunk, and absent tail. Sections revealed bilateral renal agenesis and absence of ureters and genital ducts. × 3.

FIG. 12. View of the lower trunk, showing spina bifida aperta at the sacral level. × 6.

FIG. 13. Young mouse, showing exophthalmos, absent pinna, umbilical hernia, and spina bifida aperta at the lumbrosacral level. × 3.5.

FIG. 14. Rear view, showing exencephaly and spina bifida aperta at the lumbosacral level. × 3.



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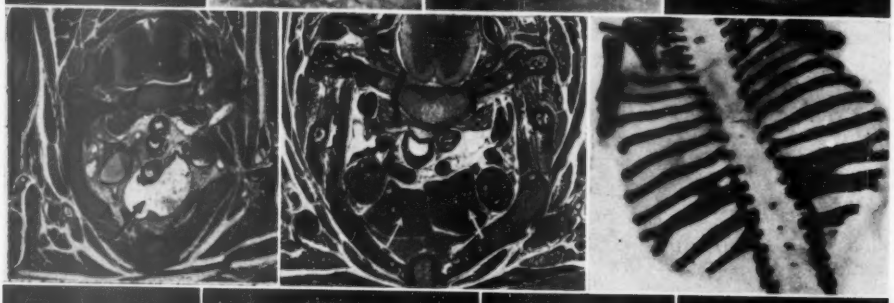

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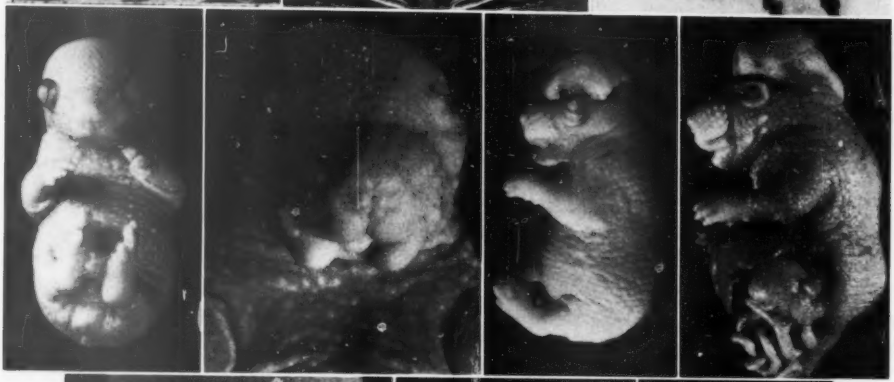

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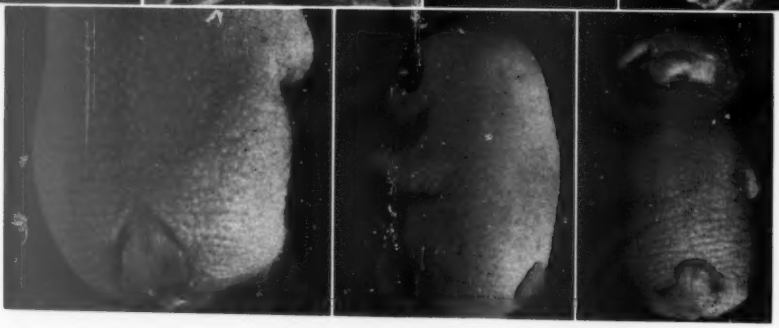

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FIG. 15. Sagittal section of a spina bifida occulta. Also seen is the crossed ectopic fused kidney. × 9.

FIG. 16. Sagittal section of a slightly protruberant spina bifida occulta. This was not noted upon external examination. × 12.

FIG. 17. Section of the chest, showing the heart with a narrow, crooked ductus arteriosus (arrow). × 17.

FIG. 18. Control. Normal ductus arteriosus. × 16.

FIG. 19. Cross section of a heart with a high interventricular septal defect (arrow). R = right ventricle; L = left ventricle. Compare with control in Figure 20. × 21.

FIG. 20. Control heart. R = right ventricle; L = left ventricle. × 21.

FIG. 21. A crossed ectopic fused kidney with a left hydroureter. The inferior vena cava and aorta lie between the midline portion of the left kidney and the vertebra. × 17.

FIG. 22. Cross section of a fused ectopic kidney with 3 ureters (just ventral to the kidney); the dilated one becomes atretic caudally; another (closest to kidney) will enter the left Wolffian duct. The bladder is moderately dilated and at its left dorsolateral border the musculature is absent. Dorsal to the bladder are two structures. The one at the animal's right is an aberrant genital duct abnormally situated. At the left is the colon, the lumen of which is about to communicate with that of the bladder. (In animals with "rectovesical" fistula. the rectum is actually absent and it is the colon that is involved in the fistula.) × 14.

FIG. 23. Midline fused kidneys with bilateral hydroureter ending blindly caudally. × 13.

FIG. 24. Midline "lump" kidney (arrow) between defective vertebrae. Ventral to it is a normal ureter and a hydroureter. Ventral to the ureters is a greatly dilated, thin-walled bladder. At the dorsal surface there is a spina bifida aperta. × 13.

FIG. 25. Frontal section through the nasopharynx (N). Above the basisphenoid is the midbrain with the pituitary beneath the hypothalamus. Lateral to N are atretic eustachian tubes in which lie ectopic thymic tissue (arrows). × 25.

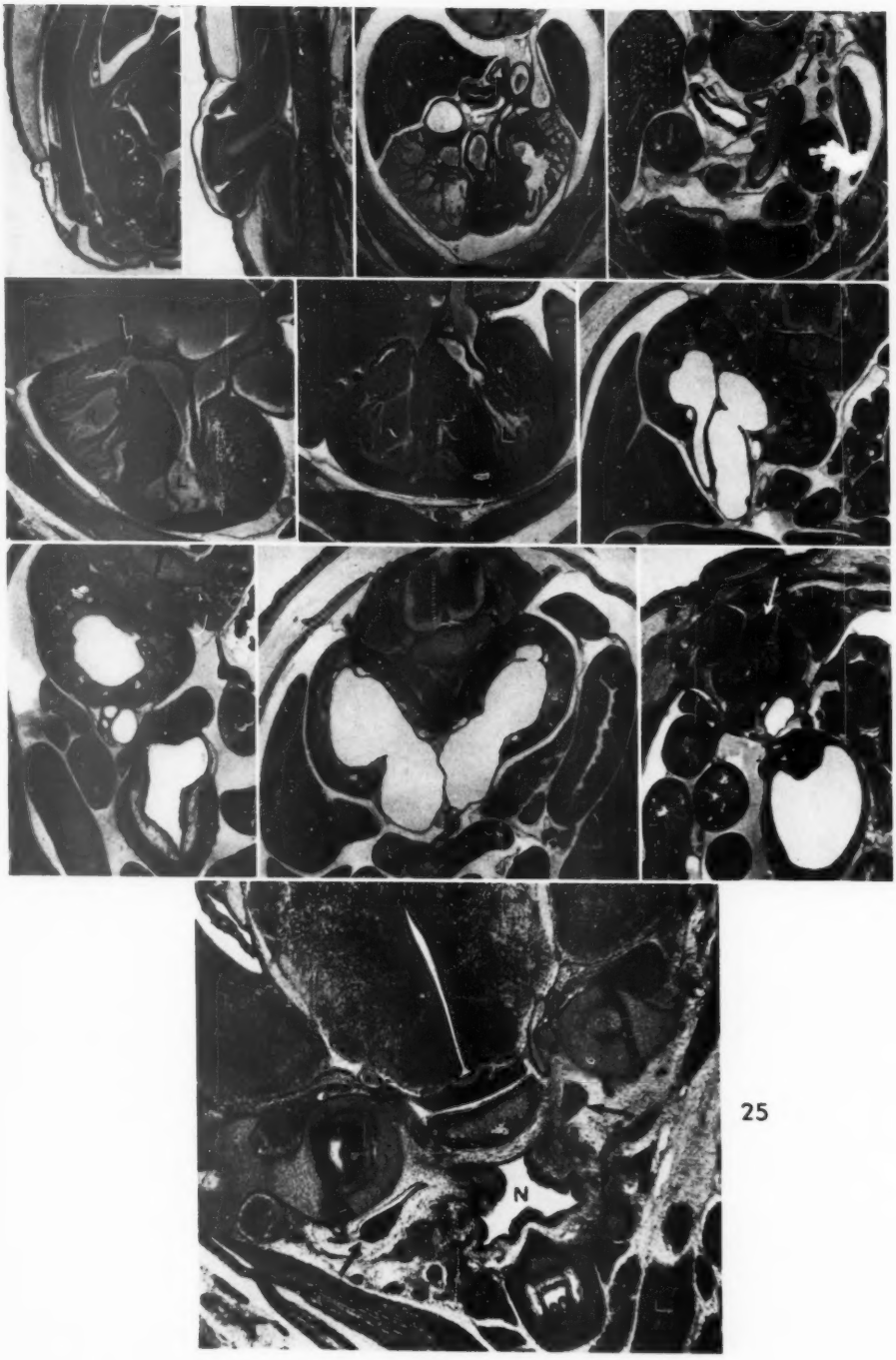
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FIG. 26. Frontal section through the mouth, showing bilateral fusion of the tongue to the gingiva, with outpouchings of the oral cavity beneath and at the sides of the tongue. × 56.

FIG. 27. Frontal section through a microstomia, showing a supernumerary lower incisor on the left (arrow). × 56.

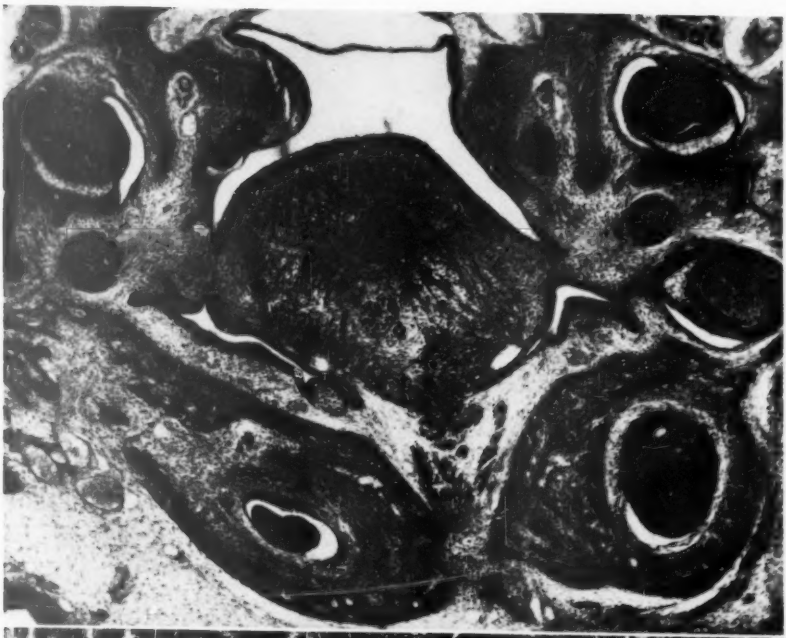

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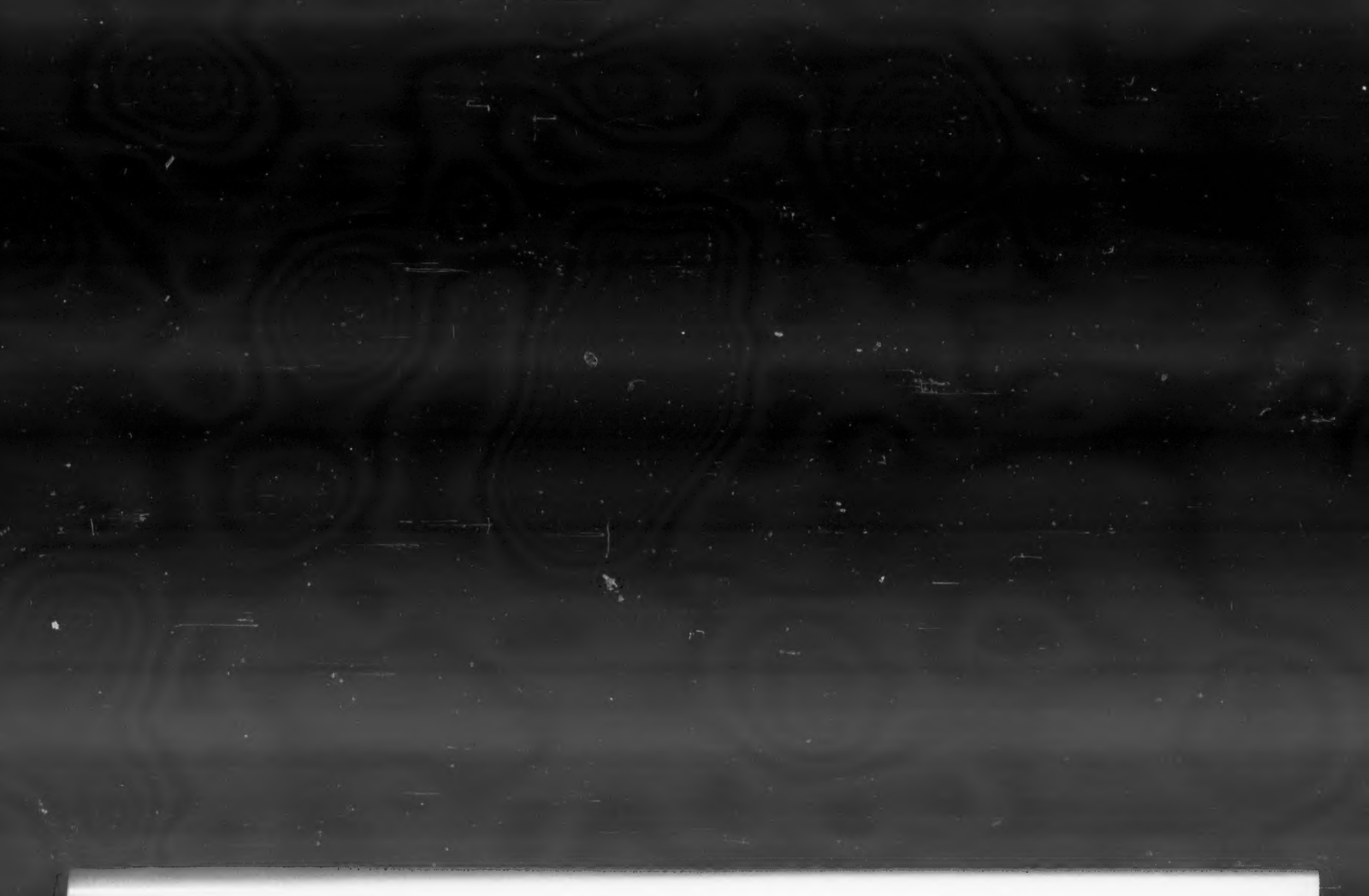
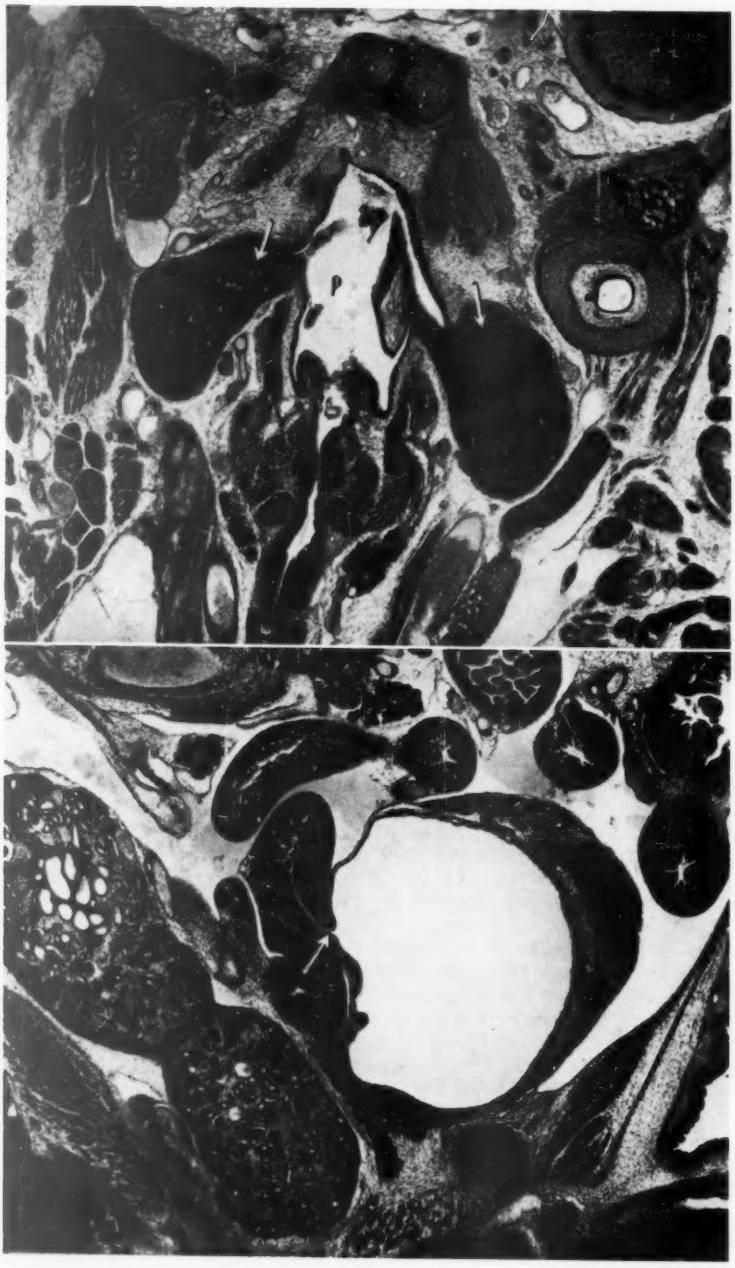
FIG. 28. Frontal section through the pharynx (P) with adjacent cervical thymuses (arrows). × 34.

FIG. 29. Sagittal section, showing dilated bladder, crossed ectopic fused kidney, and a rectovesical fistula (arrow). × 35.





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INHIBITION OF AMINONUCLEOSIDE NEPHROSIS IN RATS

I. The Effect of Adenine, Adenosine and Adenosine Triphosphate

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The mechanism involved in puromycin aminonucleoside (P-A) nephrosis is still not conclusively established. The available evidence favors the idea that the drug acts by competing with an essential precursor or inhibiting a key enzyme in the synthesis of nucleic acid.[1-3] The striking specificity of chemical structure required for nephrotoxicity is unique, since even analogues with seemingly minor alterations of the molecule are ineffective in causing the disease.[4] Similarly unique is the recent report by Hartman, Hartman and Baldridge[2] that in short-term experiments adenine partly protected animals from P-A toxicity while adenosine was totally ineffective. The present work extends these investigations, using adenine, adenosine and adenosine triphosphate (ATP) in doses relatively large compared to P-A. In addition, clinical, laboratory and pathologic observations have been extended over a period of several months following the course of drug administration.

Methods

Female rats of the Holtzman strain, weighing approximately 200 (±20) gm., were used. Since aminonucleoside (P-A) causes nephrosis in 100 per cent of treated rats, relatively few animals were required for any experiments employing P-A animals as controls. The rats were housed in individual metabolism cages in a temperature-controlled environment and were fed a diet of Fox Chow and water *ad libitum* throughout the course of the experiment. Drugs that were given once daily were administered subcutaneously in the evening, and in some cases injections were given both morning and evening. When P-A (kindly furnished by Dr. Stanton Hardy of the Lederle Laboratories) was injected with another drug, the latter was always introduced a few minutes before the former and at a separate site. Except as indicated in the footnote of Table I, injections were given daily until the day of sacrifice. P-A was dissolved in distilled water, and 0.6 ml. of an 0.5 per cent solution was injected daily. The other drugs (Nutritional Biochemicals Corporation) were dissolved or suspended in distilled water in the following concentrations: adenine, 2.5 to 5 per cent; adenosine, 2 per cent; and ATP, 10 per cent in the form of the tetrahydrate disodium salt. In order to insure and maintain stability, the ATP solution was adjusted to pH 7

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TABLE I

SHORT AND LONG TERM OBSERVATIONS ON RATS WITH AMINONUCLEOSIDE (P-A) NEPHROSIS

Group no.	Molar dose*	Day of sacrifice	Ascites (gm.)	Blood pressure (mean)†	Kidneys: Initial (gm./100 gm. body wt.)‡	Kidneys: Final (gm./100 gm. body wt.)‡	Kidneys: Water (%)	Plasma: Urea nitrogen (mg. %)	Plasma: Cholesterol (mg. %)	Plasma: Triglyceride (mg. %)	Plasma: Protein (gm. %)
					Short-term results						
Control											
1	0		0	80.0 (14)§	.385 (78)		78.48 (4)	19.1 (6)	99 (18)	24.7 (18)	6.0 (18)
				±10.3	±.010		±1.85	±1.79	±12.73	±12.7	±0.45
				70–92	.326–.449		74.30–81.20	17–21	77–123	8–52	5.4–6.8
P-A											
2	1×	9–14	5.7 (18)	73.8 (6)	.360 (11)	.388 (11)		27.0 (6)	265 (6)	76.0 (6)	5.1 (6)
			0–7	62–91	.273–.424	.359–.450		18–46	183–385	35–135	2.6–5.9
P-A + adenine											
3	7.5×	9–10	2.4 (7)		.352 (7)	.326 (7)		14 (7)	204 (7)	63	5.0 (7)
			0–7		.312–.402	.315–.404		12–16	145–297	20–102	0.9–7.6
4	10×	10	2.0 (3)		.343 (3)	.300 (3)		19 (3)	311 (3)	478 (3)	4.8 (3)
			0–6		.309–.386	.260–.388		16–23	158–504	349–679	4.4–5.7
5	20×BID	28	0 (4)	113 (2)	.881	1.090 (3)	82.4 (2)	41 (3)	234 (3)	194 (3)	5.6 (3)
				110–115	.765–1.038	.947–1.362	81.7, 83.0	37–48	179–306	59–372	4.7–5.6
Adenine											
6	10×	10	0 (3)		.343 (3)	.300 (3)		11 (3)	117 (3)	14 (3)	5.7 (3)
					.309–.380	.286–.372		10–12	90–143	3–26	5.5–6.0
7	20×BID	20	0 (2)		.623 (2)	.544 (2)	80.7 (2)	26 (2)	96 (2)	31 (2)	6.2 (2)
					.586–.660	.504–.584	80.3, 81.1	21–31	77–115	22–40	6.0–6.3
P-A+ adenosine											
8	1×	12	11.3 (3)		.416 (3)	.441 (3)		40 (3)	637 (3)	427 (3)	4.0 (3)
			8–16		.362–.468	.402–.475		39–41	593–662	318–554	3.9–4.1
9	5×	15	17.0 (3)		.386 (3)	.394 (3)		29 (3)	816 (3)	1384 (2)	5.9 (3)
			11–21		.335–.414	.335–.407		25–35	730–915	812–1955	5.0–7.2

10	10×	12	5.3 (3) 0–13		.407 (3) .369–.471	.403 (3) .382–.442		31 (2) 30–32	537 (2) 457–616	235 (2) 203–266	5.0 (2) 4.7–5.2
Adenosine											
11	7×BID	14	0 (4)		.488 (4) .422–.536	.374 (4) .358–.392	74.5 (4) 73.2–78.8	17 (4) 15–18	93 (4) 85–101	64 (4) 29–130	6.0 (4) 5.8–6.1
PA+ATP											
12	10×	11	30 (3) 24–39		.509 (4) .498–.537	.583 (4) .537–.591	84.1 (4) 83.7–84.4				
				Long-term results							
P-A											
13	1×/10	146–150	0 (4)	160 (3) 112–187	.665 (3) .528–.775	.637 (4) .427–.692	78.7 (4) 78.5–79.6	35 (3) 18–46	217 (3) 183–235	68 (3) 44–80	5.8 (3) 5.6–6.0
P-A+ adenine											
14	14×/9	102–103	0 (3)	127 (3) 121–137	.414 (3) .388–.442	.352 (3) .318–.384	77.9 (3) 77.8–78.0	16 (3) 14–17	81 (3) 64–107	11 (3) 8–16	6.0 (3) 6.0–6.0
15	22×/9	102–103	0 (3)	132 (3) 130–134	.453 (3) .407–.513	.359 (3) .312–.424	78.8 (3) 78.3–79.8	15 (3) 14–16	96 (3) 72–135	9 (3) 2–17	6.2 (3) 6.0–6.3

* P-A dose: 1.5 mg. per 100 gm. of body weight per day until sacrifice. Adenine, adenosine and ATP dose in moles times P-A dose for days indicated under day of sacrifice, except: (a) group 5, P-A given for 20 days; (b) groups 13, 14 and 15, dose and days of treatment shown as dose/days.

† Mean ± standard deviation (for controls only), and range.

‡ Body weight after removal of ascitic fluid.

§ Figures in parentheses denote the number of animals.

with 1 N NaOH and was kept frozen except to remove the daily required dose. The dose of adenine, adenosine and ATP is expressed as times molar or times moles relative to P-A. The non-sterile solutions were kept refrigerated and were warmed to body temperature prior to injection.

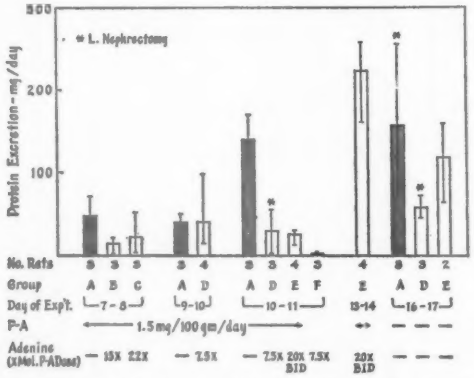
Maximum urine concentrating ability was evaluated by collecting urine overnight under oil during food and water deprivation. At the time of sacrifice animals were anesthetized with ether, weighed, and the abdominal cavity was entered through a midline incision extending from the xiphoid to the pubis. Any ascitic fluid present was removed by sponging, and the animal was reweighed. Blood pressure was measured directly at the bifurcation of the aorta with a 20-gauge needle attached to a mercury manometer by way of a 3-way stopcock and 90 mm. of polyethylene tubing of 3 mm. inside diameter. Exsanguination was carried out from the aorta into a heparinized syringe attached to one end of the stopcock.

Urea nitrogen,[5] total protein,[6] cholesterol,[7] and triglyceride [8] were determined on plasma previously frozen. Urine protein was determined by the Shevky and Stafford sedimentation method as modified by McKay,[9] and urine concentration was determined using the Fiske osmometer. Water content of the kidney was determined by slicing a whole kidney, or in some cases ½ kidney, into thin strips on a watch glass which was then dried at 100° C. for at least 24 hours.

Results

Acute Experiments

P-A plus Adenine. Adenine was given in increasing doses relative to P-A to groups B to F (Text-fig. 1). Control rats (group A), which were treated only with 3 mg. of P-A daily, developed proteinuria by the



TEXT-FIGURE 1. Effect of adenine on inhibition of P-A nephrosis. Protein excretion in rats during the acute phase.

seventh to eighth day. (Mean protein excretion for 30 normal rats was 2.2 ± 1.7 mg. per day; Text-fig. 4.) At this time 5 of the 6 adenine-P-A-treated animals (groups B and C) excreted less protein than any of the control rats, but by the ninth or tenth day the mean protein excretion was the same for both control and P-A + adenine groups. The brisk increase in proteinuria that was observed on days 10 to 11 in the

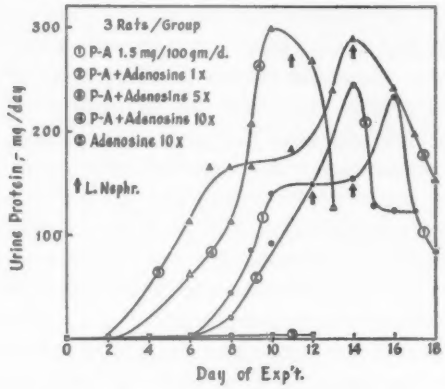
control group was somewhat delayed in the group treated twice daily with 20 times as much adenine as P-A (group E). Animals in group 3 (Table I), sacrificed on day 9 to 10, showed normal plasma urea nitrogen and less severe hyperlipemia and hypercholesteremia than the P-A controls.

Comparison of the photomicrographs of the kidney in a representative P-A-treated rat (Fig. 2) with that of an adenine-P-A-treated rat (Fig. 3) shows that in the case of the former there were occasional dilated tubules containing protein casts, and the basement membrane of an occasional glomerulus appeared thickened. In contrast, the histologic appearance of the adenine-P-A-treated animal treated for 10 days appeared normal. However, rats treated for 14 days with an even larger dose of adenine in combination with P-A showed marked proteinuria (group E, Text-fig. 1). Animals of group 5, which were treated with P-A for 20 days and adenine for an additional 8 days (total of 28 days) also showed evidence of severe nephrosis (Fig. 4). Many tubules were dilated and contained casts, and there was infiltration of neutrophils and round cells into the interstitial area. Glomeruli and Bowman's capsules were thickened with deposits of hyaline material, sometimes present in the shape of crescents. Plasma urea nitrogen, cholesterol and triglyceride were elevated above the P-A control values (Table I). The ratio of kidney weight to total body weight and the water content of the kidneys were also increased. In the situation where P-A nephrosis was already established, the addition of adenine did not reverse proteinuria except for causing a reduction in protein excretion preterminally in 2 of the animals dying from renal failure on the 16th and 17th days.

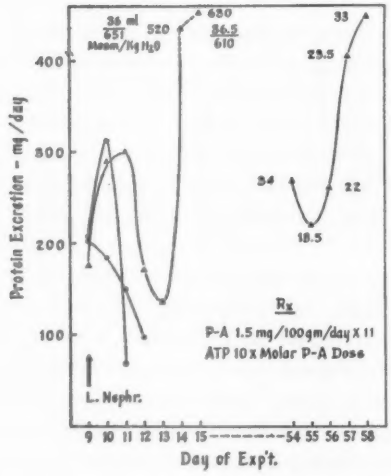
Surprisingly, adenine itself in large doses was not innocuous to the kidney. The kidneys were heavy, soft, flabby, and showed an increased water content. In addition there was a curious canary-yellow substance deposited in streaks paralleling the collecting ducts. This deposit was especially prominent in the papilla and in the outer medullary area which was sharply delineated from the cortex by a prominent white band. Microscopically, the deposit was in the form of crystalline rosettes. It was accompanied by an intense infiltration of inflammatory cells in the interstitial tissue and clumps of neutrophils in the convoluted tubules and collecting ducts. There also appeared to be proliferation of glomerular and tubular epithelial cells. The laboratory data revealed mild azotemia and slight proteinuria in 2 of the 4 rats, unaccompanied by lipid, cholesterol or serum protein changes (Table I, group 7).

P-A plus Adenosine. Adenosine in molar doses 1 to 10 times the P-A dose failed to alter the usual pattern of evolution of the disorder. All animals developed the typical features of the nephrotic syndrome (Text-

fig. 2 and Table I, groups 8, 9 and 10) and showed even more severe involvement than the control P-A kidney. No lesions were observed in the adenosine-control kidney. The elevated plasma triglyceride in the control adenosine group is significant and remains unexplained.



TEXT-FIGURE 2. Failure of adenosine to prevent proteinuria in rats with P-A-induced nephrosis.



TEXT-FIGURE 3. Severe nephrosis in rats induced by P-A + ATP combination. Only one animal survived the acute phase of the disease. Protein excretion for days 14 and 15 is 520 mg. and 630 mg., respectively, and corresponding volume and concentration are also included for these days. The figures adjacent to the graph on the right represent daily urine output.

P-A plus ATP. ATP also failed to prevent the P-A-induced nephrotic syndrome. Indeed, the pronounced ascites and markedly increased ratio of kidney weight to total body weight and water content found at the time of nephrectomy suggest that ATP may have aggravated the condition (Table I, group 12). Protein excretion, urine volume and concentration are shown in Text-figure 3. Only one animal survived the acute phase of the disorder. After unilateral nephrectomy the initial pattern of urine output and protein excretion was biphasic. Later proteinuria increased to fairly high levels and was maintained for a prolonged period in association with polyuria and impaired urine concentration.

Long Term Observation

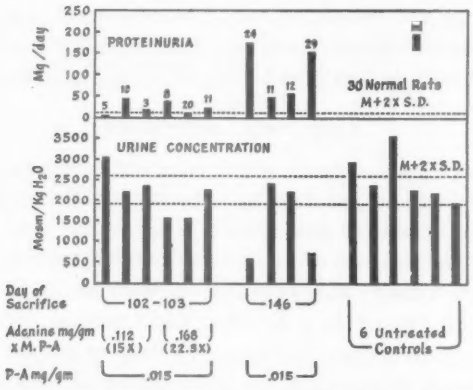
P-A Controls. Four animals which survived a 10-day course of P-A were sacrificed 140 to 150 days after treatment. However, blood pressure and blood chemistry data are available in only 3 of the survivors. None showed ascites but all 3 had moderate hypertension (Table I, group 13). The kidneys were enlarged but were not swollen; water content was normal. One of the 4 survivors had a normal plasma urea nitrogen. Figure 6 shows a severely damaged kidney of one such animal compared to a normal kidney (Fig. 5). Most glomeruli were hyalinized and basement membranes were thickened. In some there was endothelial and epithelial cell proliferation and round cell infiltration into the interstitial areas. Proximal and distal tubules were dilated and lined with flattened epithelial cells, some of which had been desquamated into the lumen. Protein casts were numerous. It is important to point out that these extensive alterations were not observed in all long-term survivors, but all showed varying degrees of degeneration.

P-A plus Adenine. The animals in groups 14 and 15 (Table I) were still in good health when sacrificed. Ascites was absent and the blood pressure was mildly elevated in all animals. In contrast to the P-A group, the kidneys were considerably smaller in size and appeared grossly normal (Fig. 7). The histologic appearance of the adenine-P-A kidney was normal, or nearly so. Plasma urea nitrogen, cholesterol, triglyceride and total protein were all normal, and were in striking contrast to the P-A control group. Insufficient time had elapsed for long-term observation on animals treated with large doses of adenine alone.

P-A plus ATP. The kidney of a representative animal sacrificed 2½ months after combined treatment with ATP and P-A is shown in Figure 8. The extent of damage was as great as or greater than the P-A-treated kidney 4½ months after treatment (Fig. 6).

Urine Concentrating Ability. Protein excretion and urine concentration on the day preceding sacrifice of normal and nephrotic rats are

shown in Text-figure 4. It is evident that 3 of 6 adenine-treated rats were able to concentrate their urine within the normal range, and 4 excreted little or no protein. In contrast, 2 of 4 P-A-nephrotic rats still demonstrated poor urine concentration, marked proteinuria and polyuria. In general, the degree of proteinuria and impaired urine concentration correlated well with the gross and microscopic alterations.



TEXT-FIGURE 4. Protein excretion, urine concentrating ability and urine output in rats with chronic renal disease induced by P-A and combined P-A + adenine treatment.

DISCUSSION

The mechanism of action of puromycin aminonucleoside (P-A) is not yet clear. The acute phase of the disorder in the first 2 weeks resembled that produced by nephrotoxic serum, whereas the lesion in the chronic P-A-affected kidney appeared less severe. However, experimental evidence to support an immune mechanism has been lacking.[10,11] Available evidence suggests that by successful competition, P-A blocks the incorporation of ribonucleic acid (RNA) precursor into pentose nucleic acid (PNA) protein.[12]

Of the nucleic acid precursors tested in this study, only adenine reduced the severity of the disease, and this occurred only when it was given early, before the full-blown disorder developed. The partial protection afforded by adenine against the acute phase of nephrosis confirmed the work of Hartman and co-workers,[2] but it is evident that our animals were not protected beyond the first week. From their data it is not possible to determine the degree of proteinuria which would have occurred had they observed their animals for a longer period. By extending the period of treatment with the adenine-P-A combination, our investigations showed that the protection afforded was only temporary and that treatment carried beyond the second week resulted in the usual

severe nephrosis and death. It is likely, however, that the slight residual kidney damage observed 3 months after P-A-adenine treatment was the result of adenine having initially lessened the damage during the acute phase.

The partial success of adenine on the one hand, and the failure of adenosine and ATP on the other, in ameliorating the P-A-induced nephrosis, may be attributed to the unique importance of adenine as a nucleic acid precursor in the rat.[13] An excess of either of the latter compounds might act in a feed-back mechanism as an inhibitor of PNA synthesis.[14]

It is interesting that the rat and mouse kidney, despite its relatively slow rate of cell turnover, has been found to have the highest ratio of PNA to deoxyribonucleic acid (DNA) of any organ as judged by the incorporation of radioactive formate.[15,16] Since the labeled DNA in the rat kidney was stable, it suggested that the basement membrane had a high PNA turnover rate, a hypothesis compatible with the regulation and transport of multiple substances passing into and out of the cell. If P-A were incorporated into the PNA of rapidly regenerating basement membrane, and further, if the presence of P-A altered its physical structure, permitting increased protein filtration or diminished reabsorption, then at least two cardinal features of the nephrotic syndrome could be accounted for—proteinuria and hypoproteinemia. The earliest electron microscopic changes observed by Vernier, Papermaster and Good at the end of the first week consisted of swelling and coalescence of epithelial foot processes anchored to the basement membrane, and this lesion concided with the appearance of proteinuria.[17]

Injury to the basement membrane may be brought about in other ways, such as through an immune reaction. The glomerular basement membrane is a much more potent antigen than glomerular capillary cells,[18] and has been found to be the site of immediate localization of injected antibodies tagged with a radioactive label[19] or fluorescein.[20,21] Perhaps the ability of the glomerular cell to regenerate normal basement membrane at a normal rate is impaired by the incorporation of antibody as it is by the incorporation of P-A. Fisher and Gruhn[22] have tabulated other similarities and differences between nephrotoxic serum and aminonucleoside nephrosis.

That adenine can produce nephrotoxicity by causing obstruction of the tubules with crystalline deposits has been known for a long time. These insoluble crystals have been shown to be 2,8-dioxyadenine and are associated with progressive renal damage.[23] The crystals appear as early as 5 hours after a 500 mg. per kg. single oral dose, and chronic changes are noted as long as 30 days later. In our studies, nephrotoxicity due to

adenine was observed only with the relatively large daily dose of 200 mg. per kg. given over a 14-day period. Clinically, the animals were not as ill as the P-A-treated group despite the comparable degree of azotemia. Nephrotoxicity due to orally administered adenine also has been demonstrated in a human subject with pernicious anemia in relapse, who was given 32.5 gm. of adenine hydrochloride over a 6-day period.[24]

The increased weight of the affected kidney in the acute phase of the nephrotic syndrome is probably a reflection of the generalized state of abnormal water retention also evident elsewhere in the body. However, the increased weight of the chronic P-A-nephrotic kidney was not due to increased water content but rather to scar tissue. In this regard, the chronically diseased P-A kidney in the rat is unlike the typical "small, scarred kidney" of chronic glomerulonephritis seen in the human adult. We have never observed the "small, scarred kidney" even in rats dying of renal insufficiency of several months' standing. Perhaps the small contracted kidney takes years rather than months to develop. The appearance of the chronically diseased P-A kidney does, however, resemble the large, pale, granular kidney of children and some adults dying after a protracted course of the nephrotic syndrome.

Summary

The effectiveness of adenine, adenosine, and ATP in preventing or reversing puromycin aminonucleoside (P-A)-induced nephrosis in rats was investigated. Adenine, but not adenosine or ATP, temporarily delayed the onset of proteinuria for a few days, but the full-blown nephrotic syndrome occurred by the second week. This substance likewise failed to reverse the course of established acute nephrosis.

Some animals were sacrificed 3 to 4 months after P-A-induced nephrosis. The kidneys of those that received adenine and P-A during the acute phase showed little residual histologic and functional damage, while kidneys of P-A-treated rats showed more severe chronic disease. Typically, the latter were large, pale, granular and firm, and resembled the kidney of protracted nephrosis in humans rather than the "small, scarred kidney" of chronic glomerulonephritis. Moderately severe hypertension occurred in animals with chronically diseased kidneys induced by P-A.

The mechanism of action of P-A is compatible with the observation that it competes with RNA precursor for PNA synthesis.

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LEGENDS FOR FIGURES

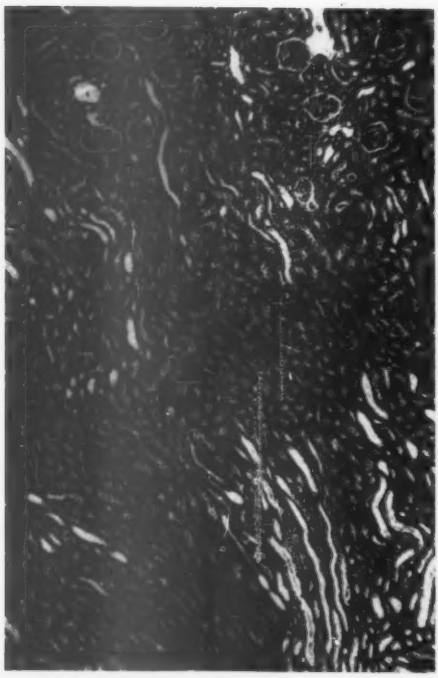
Photographs were prepared from sections stained by hematoxylin and eosin.

FIG. 1. Normal rat kidney. × 35.

FIG. 2. P-A for 10 days. There are protein casts in the tubules. × 35.

FIG. 3. P-A plus adenine, 10 times molar for 10 days. Proteinuria is less than that shown in Figure 2. × 35.

FIG. 4. Simultaneous P-A and adenine, 20 times molar, twice daily for 20 days, followed by adenine alone for 8 more days. There is marked tubular dilatation, proteinuria and enlargement of Bowman's space. The latter, however, rarely contains protein. × 48.



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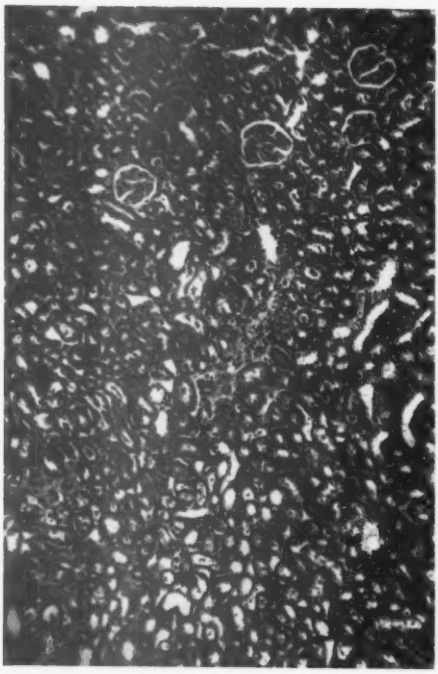

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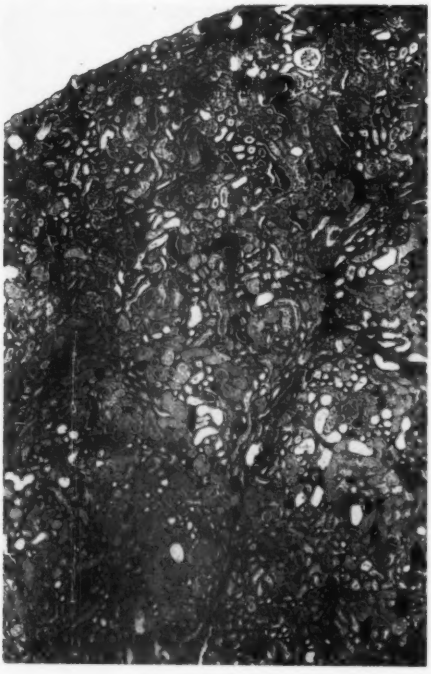

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FIG. 5. Normal rat kidney, gross and microscopic. × 35.

FIG. 6. P-A nephrotic kidney 4½ months after 10 days' initial treatment. Note extensive tubular dilatation, casts, and glomeruli in varying stages of hyalinization, and interstitial thickening. × 50.

FIG. 7. P-A plus adenine (14 times molar) nephrotic kidney 3½ months after 9 days' initial treatment. Residual changes are mild; there is some infiltration with leukocytes. × 50.

FIG. 8. P-A plus ATP (10 times molar) 2½ months after 11 days' initial treatment. Note the far-advanced destruction of glomeruli and the dilated tubules containing casts. × 30.

FIG. 9. Representative rat kidney removed from animal 3 months after receiving rabbit nephrotoxic serum—to be compared with kidneys shown in Figures 6 to 8. Nearly all glomeruli are hyalinized or absent. Interstitial area is infiltrated with inflammatory cells and the remaining tubules are dilated, some even lacking epithelium. × 30.

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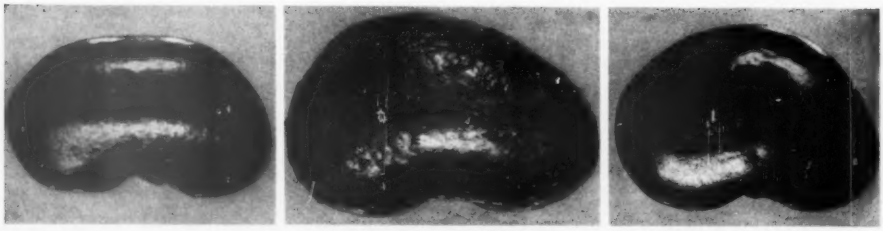

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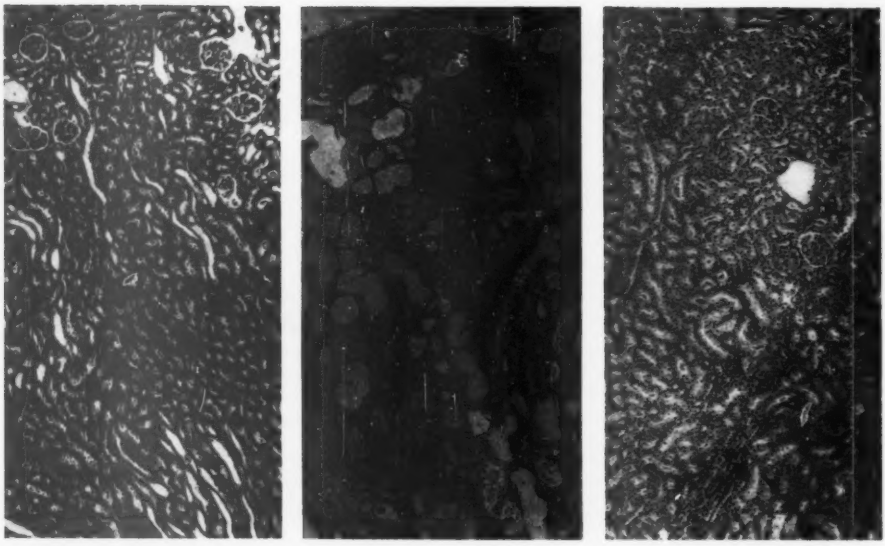

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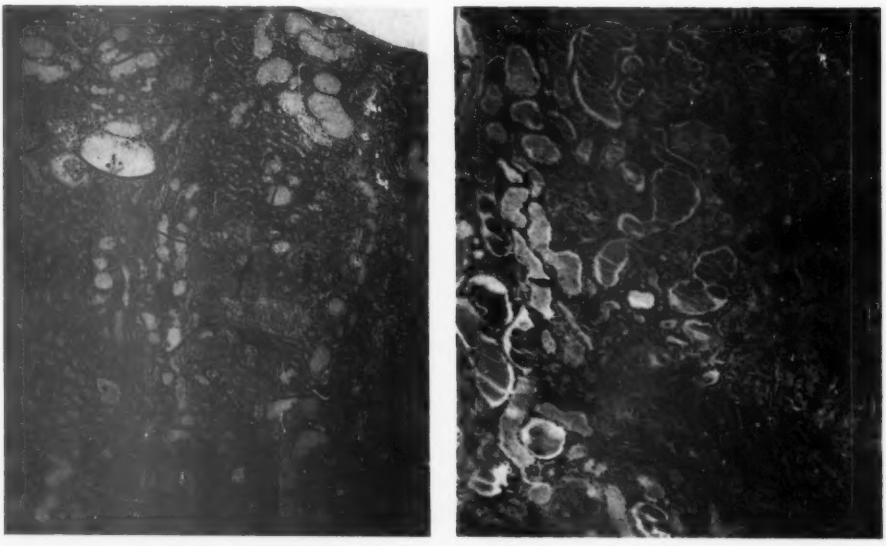

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AN IMMUNOHISTOCHEMICAL EXAMINATION OF GRANULATION TISSUE WITH GLOMERULAR AND LUNG ANTISERUMS

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A wide variety of connective tissue fibers, including lymphoid reticulin, sarcolemma, neurilemma, and most epithelial basement membranes, may be demonstrated by the so-called silver impregnation reticulin stains. Cruickshank and Hill,[1,2] using the fluorescent antibody technique of Coons and Kaplan,[3] showed that these structures apparently shared a common antigen (or antigens) as demonstrated by *in vitro* examination of rat tissues with antibody conjugates prepared against rat glomeruli and lung. This antigen, however, was not found in mature collagen. During fibrillogenesis, the immature fibers that first appear can also be demonstrated by the methods of silver salt impregnation, and hence are referred to usually as reticulin fibers; as they mature to collagen, this staining capacity is lost.

The present investigation was designed to ascertain whether the immature fibers of granulation tissue might be so constituted as to be antigenically similar to those which were found to react specifically with the anti-glomerular and anti-lung conjugates.

Material and Methods

Preparation of Antigens and Antiserums

Renal glomeruli from adult Wistar rats were isolated according to the method of Krakower and Greenspon.[4] The isolated glomeruli were subjected to sonic vibration for 20 minutes in a Raytheon 10 kc. oscillator and the washed fibrillar material suspended in isotonic saline to a concentration of 100 mg. per ml.

Lung tissue from the same animals was finely minced in a Waring blender, kept cool in an ice mixture, and the minced tissues subjected to sonic vibration. After being washed with isotonic saline several times, the centrifuged deposit was suspended in saline to a concentration of 350 mg. per ml.

The antigens were emulsified with 2 parts of kidney or lung suspension and 3 parts of incomplete Freund adjuvant (Difco Laboratories, Detroit, Michigan). Four interscapular subcutaneous injections of either the lung or the kidney emulsified antigen were given to 2.5 kg. rabbits at weekly intervals. Altogether 200 mg. of glomerular antigen or 700 mg. of lung antigen were injected into any given rabbit. The animals were bled 4 weeks after the last injection.

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* National Research Council Fellow.

The methods used for extracting the globulins from the antiserums, conjugating them with fluorescein isocyanate, preparing tissue sections, staining them and photographing the tissue reactions have been described in detail in a previous paper.[5]

Tissues Examined

A number of rat tissues (kidney, heart, skeletal muscle, lung, brain, peripheral nerves, liver, spleen, lymph nodes, skin, thyroid, pancreas, adrenal and ovary) were examined *in vitro* with the conjugated antiserums.

Granulation tissue was produced in rats about subcutaneously implanted gelfoam pledgets, as previously described.[6] These animals were sacrificed 1, 2, 3, 4, 7 and 9 days later. Frozen sections were prepared in a cryostat from the zones of granulation tissue and examined immunohistochemically with conjugates of both the glomerular and lung antiserums. After photographic records were made, the sections were washed in buffer, and restained with hematoxylin and eosin. Each frozen block of tissue from which the above sections had been obtained was then removed from the cryostat, fixed in formol saline, and paraffin sections were stained by Gomori's reticulin and silver methenamine methods. This allowed a comparison of the same granulation tissue area by these various stains. It was found that poor staining results were obtained with the silver impregnation methods if serial frozen sections were used.

In Vivo Experiment

Other rats received intravenous injections, 48 hours after the pledget had been implanted, with 1.5 ml. of lung or glomerular antiserum (the same antiserums used for preparing the conjugates described above). These rats were sacrificed 24 hours after the intravenous injection (granulation tissue, 72 hours' duration), and an attempt was made to trace the distribution of the injected antiserums by treating a variety of tissue sections, including the zones of granulation tissue, with anti-rabbit globulin prepared in sheep and conjugated with fluorescein (Sylvana Chemical Company, Orange, New Jersey).

Controls for Immunohistochemical Specificity

In Vitro Experiments. Serial cryostat sections were treated as follows: (1) Stained with conjugated antiserum. (2) Conjugated normal rabbit globulin. (3) Conjugated antiserum previously absorbed with the tissue extract (antigen) used in preparation of the antiserum. (4) Coons's slide blocking technique.[7] (5) Unstained section.

The reaction was considered to be specific if only Slide 1 showed positive fluorescence when examined by ultraviolet microscopy. Control (3) invariably caused complete inhibition of fluorescence, while the Coons technique (4) caused either complete or markedly diminished fluorescence.

In Vivo Experiments. Coons's slide blocking technique only was used.

Results

In Vitro Experiments

Examination of the various rat tissues, mentioned above, with the fluorescein conjugates of both rat glomerular and lung antiserums, revealed a specific fluorescence with a wide variety of connective tissue fibrils such as epithelial basement membranes, capillaries, sarcolemma, neurilemma and lymphoid reticulin (Figs. 1 and 2). Identical results were observed in each tissue when examined with either of the conjugates. These findings directly confirm the original observations of Cruickshank and Hill,[1,2] who believed that they indicated the presence

of a common antigen (or antigens) in the reacting tissues. It should be noted that we did not observe any specific fluorescence in renal epithelium. This had been noted by Cruickshank and Hill when they used "whole kidney" antiserum but not with glomerular antiserum.

When the zones of granulation tissue about the pledgets were examined with the same antiserum conjugates, one invariably could observe specific fluorescence in newly formed capillaries from the time of their first appearance (Figs. 4 and 6), and it continued to be present in the maturing vessels up to the longest period of observation, namely, 9 days.

Examination of the same capillaries when the slide was restained by hematoxylin and eosin showed that the reacting vessels were plump and cellular (Figs. 5 and 7), and comparison with adjacent sections from the same block stained for reticulin and by the silver methenamine method gave one the impression that more than the capillary basement membranes was staining; it appeared that the reacting antigen also was present in or on the endothelial cytoplasm (Figs. 6 and 7).

In no instance was any specific fluorescence observed in the many fine immature reticulin fibrils that were prominent in neighboring sections stained by Gomori's reticulin method (Figs. 8 and 9). These fibers only showed a nonspecific autofluorescence which became somewhat more prominent as they matured.

In Vivo Experiments

The glomerular antiserum that was injected intravenously proved to be biologically active as judged by the quantitative production of proteinuria. Examination of kidney sections with the fluorescent anti-rabbit globulin showed a brilliant specific fluorescence limited to the glomerular basement membrane, indicating that the injected antiserum had united with an antigen in this location (Fig. 3). On the other hand, when granulation tissue about the pledgets was examined in a similar manner, no fluorescence was observed in either the capillaries or immature fibrils; nor could we demonstrate any specific fluorescence in a variety of other tissues that had shown a positive reaction in the *in vitro* experiment, e.g., various connective tissue fibrils and capillaries in skeletal muscle, heart, spleen, intestine, liver, adrenal, ovary, etc.

Although the lung antiserum conjugate gave identical *in vitro* fluorescent staining reactions to those observed with the antiglomerular conjugate, the lung antiserum did not produce significant proteinuria when it was injected intravenously. Examination of the kidneys from these animals with the anti-rabbit globulin conjugate, again showed specific fluorescence confined to the glomerular basement membrane. However, the degree of fluorescence was not nearly so brilliant as that observed

with the injected antiglomerular serum. These results probably indicate that the latter was a higher titer antiserum than the former. Again, no specific fluorescence was observed in the granulation tissue or in the other tissues examined.

Discussion

The observations reported above directly confirmed the original work of Cruickshank and Hill [1,2] who showed that various naturally occurring connective tissue fibrils apparently shared a common antigen (or antigens) that reacted with both anti-glomerular and anti-lung fluorescein conjugates. These various fibrillar elements, with the exception of the renal glomerular basement membranes, are argyrophilic and hence have been classified as reticulin.

The present investigation has shown that the anti-glomerular and anti-lung conjugates that had reacted with the reticulin in a wide variety of organs, failed to react with the early argyrophilic fibers in areas of fibrillogenesis. This would indicate that the latter fibers were antigenically dissimilar to the other naturally occurring reticulins or, less likely, the concentration of antigen in them was too low to be observed by this method. This observation agrees with the concept of Robb-Smith [8] who has recently summarized the work from his and other laboratories.[9,10] This has done much to clarify the confusion that envelops the term "reticulin." It is his contention that while basement membrane reticulin and the immature argyrophilic collagen fibers (usually referred to as reticulin) share certain common features, there are also distinct differences between them. Thus, the former is an extremely stable glycolipoprotein which is not a precursor of mature collagen, whereas the latter are readily soluble in sodium chloride and citrate buffer and during fibrillogenesis appear to be incorporated into and replaced by the collagen fibers. The observations reported in the present investigation would indicate that they differ antigenically as well.

Capillaries reacted specifically with both the anti-glomerular and anti-lung conjugates in all tissues examined, including the early zones of granulation tissue. The apparent thickness of the fluorescing immature and cellular capillaries in the latter, and the presence of nuclear shadows in the zones of fluorescence suggest that the reacting antibody was united to an antigenic substance related to the endothelial cytoplasm; the reaction did not appear to be limited to the capillary basement membrane *per se*. The investigation, as performed, does not allow one to specify whether this capillary antigen is identical to that in the basement membrane reticulins, or whether it was visualized by union with

some other antibody present in the conjugate that had been prepared against the relatively crude antigenic tissue extracts.

In this connection it should be noted that Pressman and colleagues[11,12] have reported that antibodies produced against kidney, lung, and liver are probably directed against antigens contained in their vascular beds and, to some extent, these antibodies are organ specific. Certainly the antibody conjugates in the present investigation were shown to react with capillaries wherever they existed.

When the glomerular and lung antiserums were injected intravenously, the only site of union with antigen that could be demonstrated was in the glomerular basement membranes. No union was observed in the other tissue sites where an *in vitro* reaction was readily demonstrated. The dissimilarity of the observations made *in vivo* and in particular the avidity of the glomeruli for injected anti-organ serums are vexing problems that remain to be solved. A discussion of these is not within the scope of this paper, but some aspects have been covered recently by Krakower and Greenspon,[13] Seegal,[14] and Hiramoto, Jurandowski, Bernecky and Pressman.[15]

Summary

Fluorescein antibody conjugates, prepared against rat glomeruli and lungs, reacted *in vitro* with a wide variety of naturally occurring reticulins but failed to react with the young argyrophilic fibers (reticulin) in areas of granulation tissue. This would indicate a lack of antigenic relationship between these groups of argyrophilic fibers which also differ in some respects chemically. The same conjugates reacted specifically with capillaries in many organs and with newly formed capillaries in the areas of granulation tissue. The reacting antigen seemed to be associated with the cytoplasm of the young capillary endothelial cells rather than with the basement membranes *per se*. When the same unconjugated antiserums were injected intravenously, the only demonstrable site of localization was in the glomerular basement membranes.

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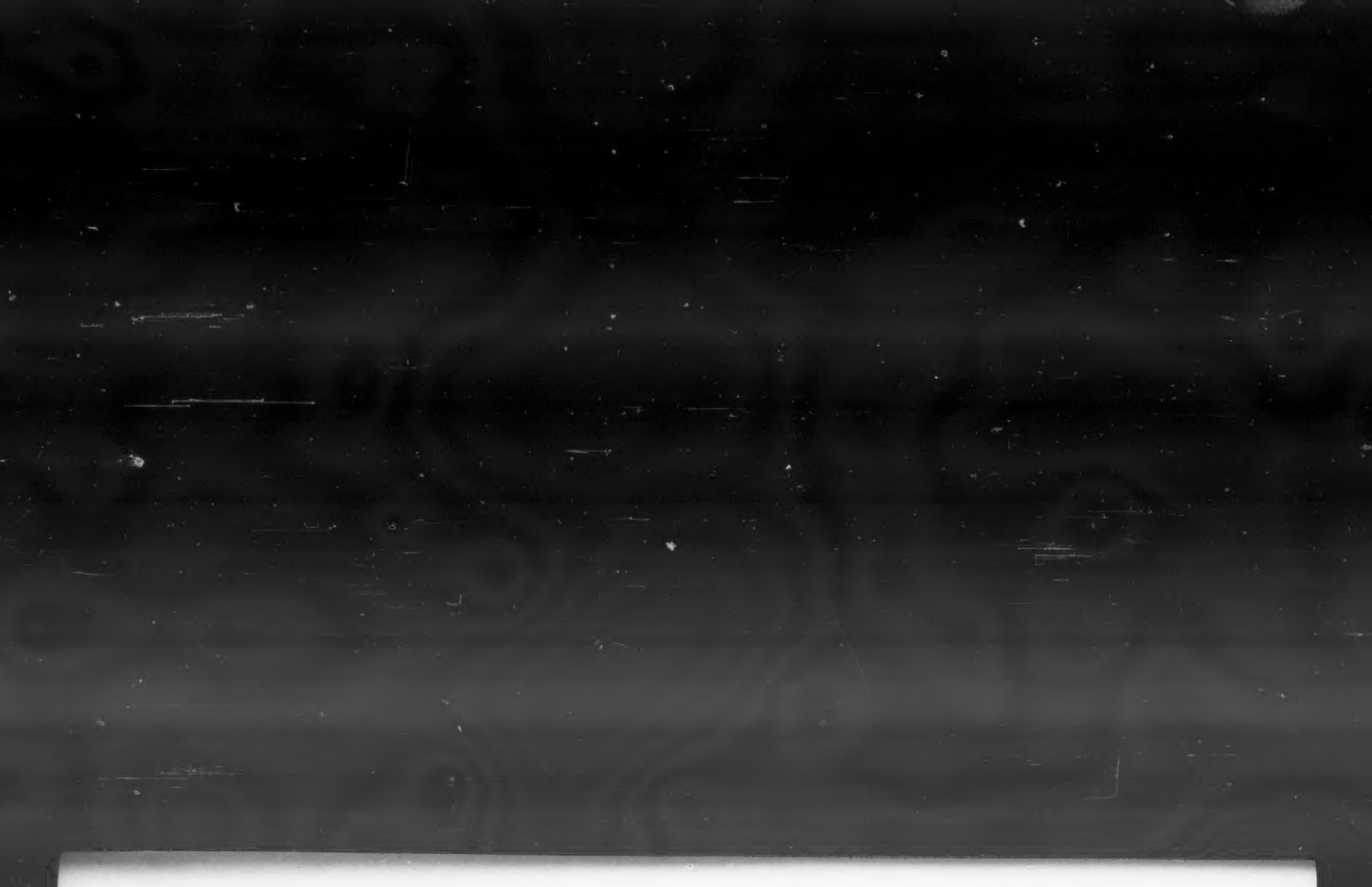
We wish to acknowledge the assistance and enthusiasm of C. F. A. Culling and B. J. Twaites who performed the technical work of this investigation.

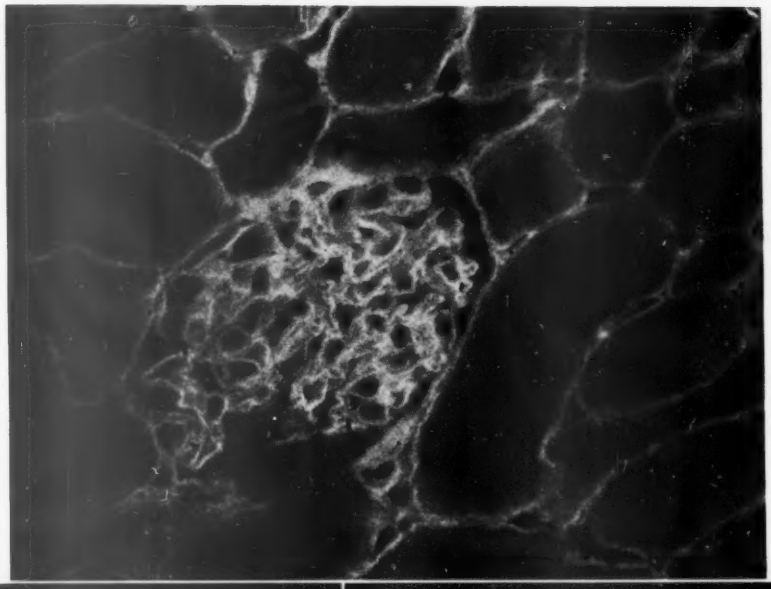
LEGENDS FOR FIGURES

FIG. 1. Fluorescent microphotograph of a rat kidney stained *in vitro* with rat anti-lung fluorescein conjugate. Glomerular and tubular basement membranes as well as intertubular capillaries show specific fluorescence. Identical results were obtained with rat anti-glomerular conjugates. × 500.

FIG. 2. Rat pancreas similarly stained. The alveolar basement membranes show specific fluorescence as do the capillaries which are shown well in the islet of Langerhans, upper left. × 500.

FIG. 3. Kidney in a rat given rat-glomerular antiserum intravenously. The antiserum was prepared in a rabbit, and the kidney was stained with fluorescent anti-rabbit globulin. The *in vivo* glomerular antiserum has only united with the glomerular basement membrane. Compare with Figure 1. × 750.





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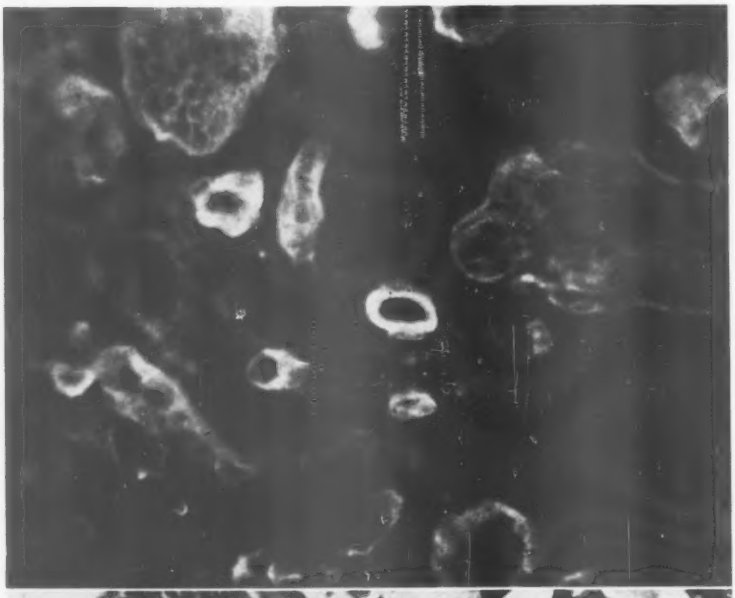

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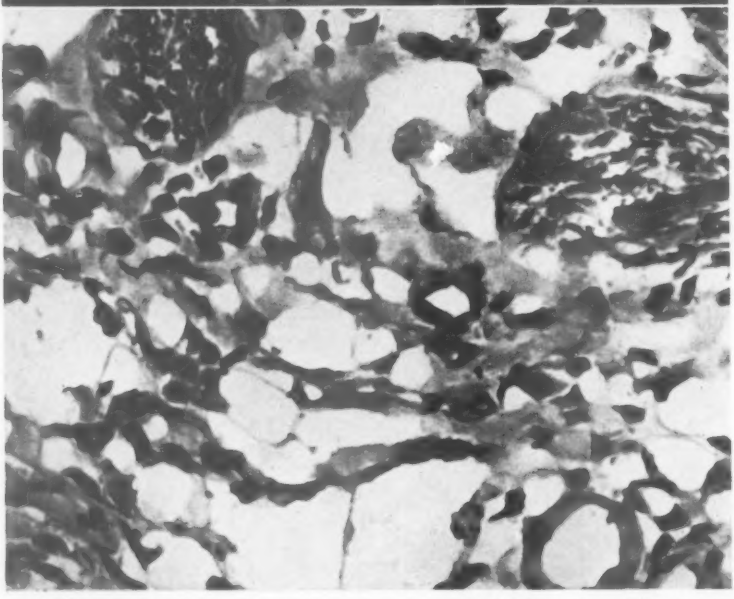
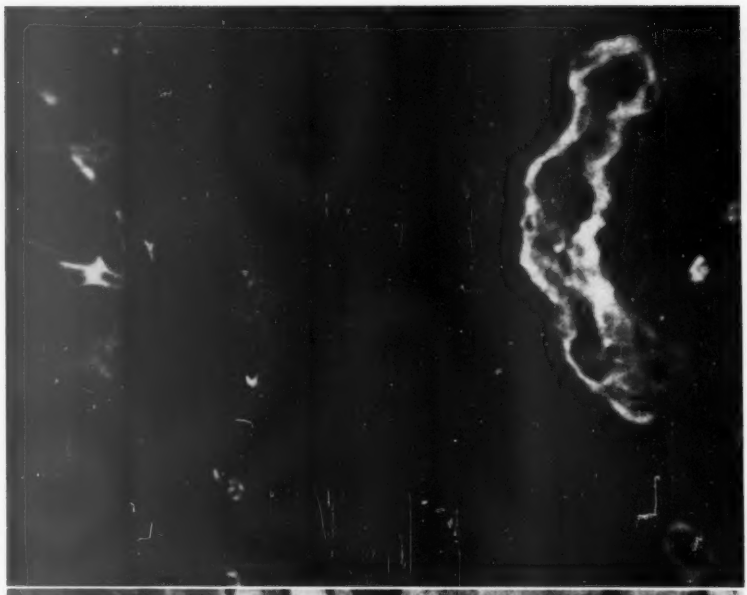


FIG. 4. Granulation tissue after 48 hours from a rat, stained *in vitro* with rat antiglomerular fluorescein conjugate. The two larger structures, in the upper left and right, are nerves in which the neurilemma shows specific fluorescence. Multiple newly formed capillaries also show a specific reaction. × 500.

FIG. 5. The same section as Figure 4 having been washed in buffer and restained with hematoxylin and eosin. By comparing this illustration with Figure 4, one can identify the immature cellular capillaries that showed the specific fluorescence. The nerves are also shown. × 500.



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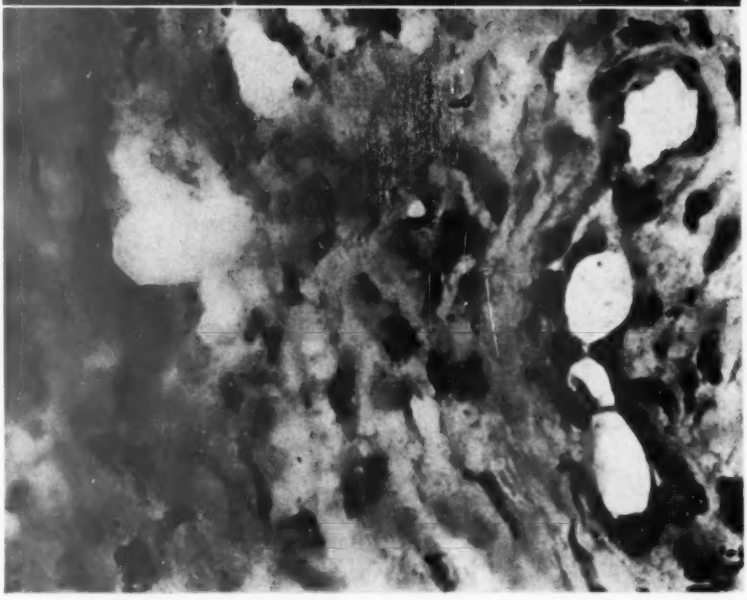

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FIG. 6. Granulation tissue after 96 hours, stained *in vitro* with rat anti-lung fluorescein conjugate. The capillary on the right shows specific fluorescence as does the sarcolemma of skeletal muscle just seen on the far left. The fibrils barely visible in the background show nonspecific autofluorescence. × 500.

FIG. 7. The same section as in Figure 6 restained with hematoxylin and eosin. Comparison enables one to identify nuclear shadows in the fluorescent capillary. These shadows are seen best on the upper half of the fluorescent microphotograph, and their presence suggests that the reacting antigen, which has combined with the fluorescent antiserum, is present in the endothelial cytoplasm. × 750.

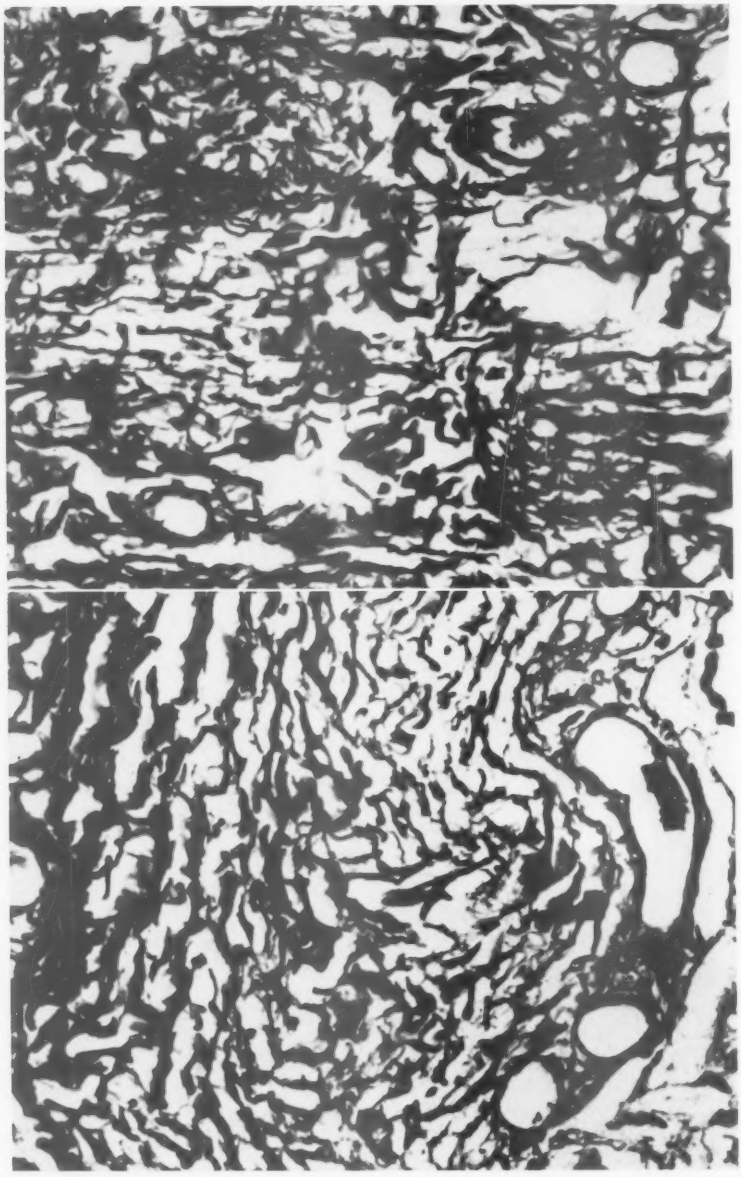
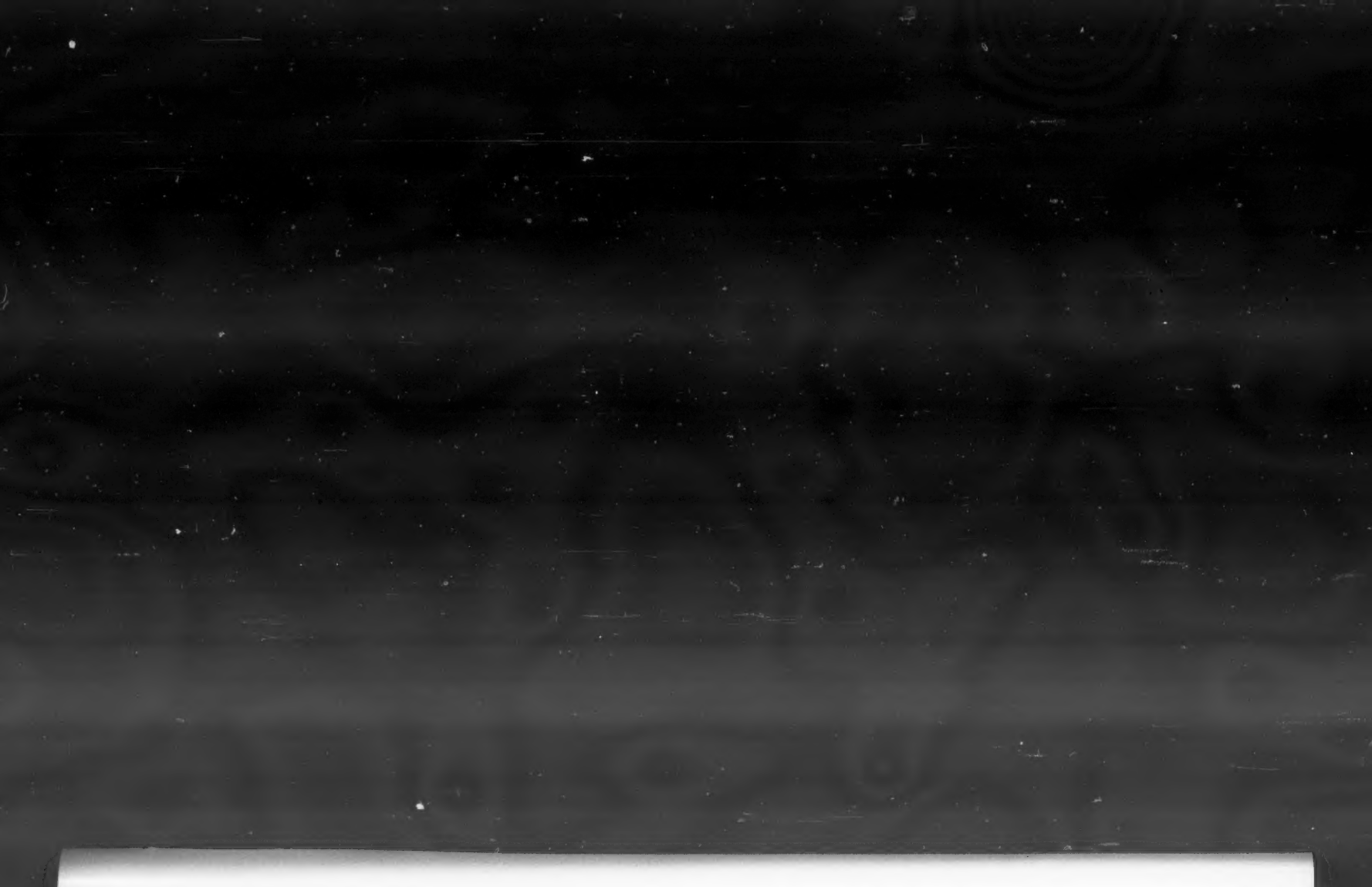


FIG. 8. The same general area of 48-hour granulation tissue shown in Figures 4 and 5. Note the large numbers of argyrophilic "reticulin" fibers which had not reacted with the fluorescent antiserums. Gomori's reticulin stain. × 650.

FIG. 9. Granulation tissue after 96 hours, again showing numerous argyrophilic fibers. Same area as that shown in Figures 6 and 7. Gomori's reticulin stain. × 650.



AMYLOIDOSIS OF THE ISLETS OF LANGERHANS

A Restudy of Islet Hyalin in Diabetic and Nondiabetic Individuals

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Hyalinosis of the islets of Langerhans in diabetes mellitus was reported for the first time by Opie in 1901.[1a,1b] He interpreted it as a degenerative change related to the pathologic physiology of the islets in this disease.[1b] This hypothesis was soon challenged by Ohlmacher,[2] Cecil,[3] and later Wright[4] and others, who reported the occurrence of hyaline deposits in the islets of nondiabetic patients. In these reports, the absence of diabetes was a presumption unsupported by glucose tolerance tests. Recent studies by Bell[5] disclosed a ratio of incidence of hyaline islets in patients with diabetes to those without it of 3 to 18:1 (depending on age groups). He re-affirmed the relationship of this islet change to diabetes mellitus and suggested that nondiabetic individuals presenting this alteration might represent instances of potential or unrecognized diabetes mellitus.

There have been several studies attempting to define the nature of the hyaline substance. Opie[1b] was struck by its histologic resemblance to amyloid but could not confirm this with amyloid stains. Mallory[6] thought that the deposit was closely related to amyloid. Warren and LeCompte,[7] at Mallory's suggestion, used methyl violet and iodine green and obtained positive stains in 14 of 51 cases of diabetes mellitus with hyaline islets. Gomori[8] stated that amyloidosis of the islets of Langerhans occurred occasionally in diabetes. Ahronheim[9] demonstrated metachromasia with gentian violet in all of his cases with hyaline islets (67 with diabetes and 5 without this disorder). Arey[10] demonstrated positive methyl violet staining for amyloid in all of 42 cases with and without diabetes, showing prominent hyalin in the islets of Langerhans. These investigators did not report results with Congo red. Warren and LeCompte,[7] Bell,[11] and Hartroft,[12] as well as standard works in pathology, continue to refer to the islet material as hyalin.

We have undertaken to restudy the nature of the hyaline material by the use of more elaborate histologic techniques than previously employed.

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Material and Methods

Sections of head, body and tail of the pancreas were taken as soon after the start of a necropsy as possible. These were immediately fixed in Kaiserling I. In selected cases, specimens were also fixed in Bouin's solution and formol-calcium.

Sections of pancreas from 91 cases of diabetes mellitus over the age of 50 were examined. As controls, sections of pancreas from 178 patients over the age of 60 years and not known to have diabetes mellitus were also investigated.

Sections (paraffin, unless otherwise specified) were stained with hematoxylin and eosin, Gomori's trichrome, Goldner's phosphotungstic acid hematoxylin (PTAH), silver (author's—JCE—modification of Bielschowsky's method), von Kossa, Sudan IV (frozen sections), toluidine blue, Alcian blue, Congo red (paraffin and frozen sections), crystal violet, and iodine green. Representative sections were examined in polarized light before and after staining with crystal violet, Congo red, and others. The crystal violet and Congo red stains were prepared in all our cases of islet hyalinosis; the remaining techniques were carried out in instances selected for excellence of preservation and fixation.

As controls for amyloid, sections of atrium, kidney, and spleen from cases of generalized secondary amyloidosis were examined, using all of the stains and histologic techniques listed above. Additional control tissue was obtained from cases of primary systemic amyloidosis, senile cardiac amyloidosis, and localized cutaneous amyloidosis in skin tumors.

Sections of left atrium from 20 cases which showed hyalin in the islets of Langerhans were stained with Congo red and examined for the presence of amyloid.

Results

Hyaline material was found in the islets of Langerhans in 45 of the 91 cases of diabetes mellitus examined. It was also encountered in the pancreatic sections from 7 of 178 patients not known to be diabetic (Table I). In the latter group, there were 3 patients in whom the existence of diabetes mellitus was presumed on the basis of single blood sugar determinations and family history. As recorded in Table II, all hyaline deposits in islets exhibited crystal violet metachromasia and Congo red binding (Fig. 3); positive birefringence and dichroism in polarized light were observed after staining with Congo red (Fig. 4). This phenomenon was not observed in unstained tissue or after the use of stains other than Congo red. The homogeneous islet substance showed positive staining with Alcian blue and the periodic acid-Schiff (PAS) stain; it was negative with toluidine blue.

Examination of Table II shows that the islet deposit and amyloid controls reacted in identical fashion with the techniques employed. The only exception was a negative reaction with the Alcian blue stain on secondary amyloid. Amyloid was not demonstrated in the atrial walls or kidneys of any of the cases examined. The incidence of diabetic glomerulosclerosis in the 45 patients with diabetes who showed hyalinized islets was approximately the same as in the remaining 46 cases of diabetes without hyalinized islets. The hyaline material was found (as

previously described) between capillary walls and islet cells (Fig. 1). With silver stains, the relationship of the hyalin to argyrophilic fibers was as follows: (1) Occasionally it lay between capillary endothelium and argyrophilic fibers. (2) Occasionally it appeared between argyrophilic fibers and islet cells. (3) In some instances it was noted to be within the argyrophilic network, spreading apart individual fibers or groups of fibers and occupying the interstices of the distended network (Fig. 2).

TABLE I

HYALINOSIS OF ISLETS OF LANGERHANS.
NECROPSY INCIDENCE

	Diabetic patients over age 50	Nondiabetic patients over age 60
Total number of cases	91*	178
Number of cases with hyalinosis of islets	45† (49.5%)	7 (3.9%)

* Excludes 4 cases of juvenile diabetes, 1 case of hemochromatosis, and 1 case of diabetes associated with carcinoma of the body of the pancreas.

† Two cases show partial involvement of a single islet.

TABLE II

HYALINOSIS OF ISLETS OF LANGERHANS.
STAINS FOR AMYLOID

	Islet hyalinosis				Amyloid controls			
	Diabetes mellitus (45 cases)		"Nondiabetic" (7 cases)					
Stain	No. pos.	No. neg.	No. pos.	No. neg.	Secondary	Primary	Senile cardiac	Local cutaneous
Metachromasia with crystal violet	45	0	7	0	+	+	+	+
Congo red	45	0	7	0	+	+	+	+
Dichroic birefringence after staining with Congo red	+		+		+	+	+	+
Periodic acid-Schiff	Mod. +		Mod. +		Mod. +	Mod. +	Mod. +	Mod. +
Toluidine blue	−		−		−	−	−	−
Alcian blue	+		+		−	+	+	+

There was no predilection of the hyalin for any part of the pancreas (head, body or tail) except insofar as there were more islets present in the tail region.[18] The deposit was found with equal frequency in intralobular and periductal islets of Langerhans. In 3 cases (2 diabetic, 1 nondiabetic) the hyaline material was observed to contain pale bluish granules (Fig. 3) when stained with hematoxylin and eosin. The von Kossa reaction confirmed the calcific nature of these granules.

Discussion

The results of this study lead to the inevitable conclusion that the hyaline material in the islets of Langerhans is amyloid.

Amyloid is a hyaline substance which stains metachromatically with the rosaniline group of dyes and which binds Congo red. It is known to consist chiefly of protein plus a small carbohydrate component which includes acid mucopolysaccharide.[14] The mucopolysaccharide fraction is responsible for the metachromasia with rosaniline dyes as well as the staining with Alcian blue and PAS.[15–17] Arey[10] and Ahronheim[9] also found metachromasia with the rosaniline dyes in all their cases of islet hyalinosis. These investigators did not report results with Congo red. Congo red staining was noted by Bloom[18] in hyalinized islets in a cat with spontaneous diabetes mellitus, and more recently Seifert[19] reported positive Congo red stain of islet hyalin in human diabetes. Seifert did not mention the number of cases studied or the percentage with Congo red positivity. He concluded that the islet material was predominantly an acid mucopolysaccharide and, therefore, not an amyloid. This conclusion was based on the staining reaction with Alcian blue and astral blue. There is no evidence, however, that positive staining with Alcian blue necessarily indicates a predominance of acid mucopolysaccharide in the substance stained. Amyloid, which is known to consist chiefly of protein, reacts in positive manner to Alcian blue (senile, primary and cutaneous types[17]). The islet hyalin in our cases stained with Alcian blue. We interpreted this to mean only that an acid mucopolysaccharide fraction was present. Rinehart, Toreson and Abul-Haj[20] established the presence of acid mucopolysaccharide in islet hyalin by the use of a colloidal iron preparation. The demonstration of carbohydrate in islet material by these methods is consistent with the general histochemical behavior of amyloid.

The basis for Congo red binding has not, to our knowledge, been explained. Unstained amyloid may show very weak birefringence[21] in discontinuous fashion, probably depending upon fortuitous arrangement of amyloid fibrils in parallel bundles. After Congo red staining, anisotropism is exaggerated[15,21–23] and readily observed with ordinary polarizing filters (Figs. 3 and 4). Birefringence of amyloid has also been noted after staining with chlorantin fast red and with Evans blue.[15] The birefringence in polarized light after Congo red staining is dichroic,[15,22,23] i.e., two different colors appear in the polarized field. Amyloid bands are green in one plane and yellow-pink in the other. Rotation of the specimen through an angle of 90° reverses the colors. This phenomenon has been attributed to parallel orientation of dye

molecules along fibers and has been described by Pearse as characteristic, but not specific, for amyloid.[15] It has also been observed, for example, in cellulose fibers dyed with Congo red.[15] The fibrillar nature of amyloid has been demonstrated by electron microscopic studies of experimental as well as human amyloid.[24–26] In a recent study of experimental amyloid in the mouse, Gueft and Ghidoni[26] showed that the fibers exhibited characteristic periodicity. It is not yet known if this periodicity is the same in amyloid substances in other animals or in human subjects.

As noted in Table II, the hyaline deposits in islets in our cases also exhibited dichroic birefringence after staining with Congo red; this has not been reported previously. Study of the fine structure of islet amyloid is subject to the practical difficulty of obtaining suitably fixed human specimens.

Calcium deposition in islet hyalin (Fig. 3) has been reported to be rare.[7] However, it occurred in approximately 6 per cent of our cases (3 of 45). Calcification of primary and secondary amyloid is not rare.[27]

The presence of amyloid in the islets of Langerhans in this series was not accompanied by amyloid deposits in any of the other organs examined. The blood vessels of the pancreas did not contain this substance, and none was found in sections of the left atrium and kidney when examined by special stains.* The pancreas does not often participate in systemic amyloidosis; when present, amyloid deposits are generally restricted to the walls of small blood vessels.[29] Islet involvement in generalized primary or secondary amyloidosis is uncommon; Ahronheim[9] found small amyloid deposits in islets in 2 of 11 cases with systemic amyloidosis. Dahlin[30] reported slight involvement of islet capillaries in 3 of 30 cases of secondary amyloidosis. Buerger and Braunstein, in a recent report of so-called senile cardiac amyloid, found deposits in the islets in 7 of their 33 cases.[31] However, their material was not studied from the standpoint of the incidence of diabetes mellitus.[32] We were unable to demonstrate amyloid in sections of the left atrium in 20 of our cases with amyloidosis of the islets of Langerhans.

Therefore, amyloid in the islets of Langerhans constitutes a form of localized amyloidosis. Localized amyloid without systemic deposition is known to occur in the stroma of certain cutaneous lesions,[33,34] particularly basal cell papilloma and basal cell epithelioma. Other reported

* According to Fahr,[28] the lesions of diabetic glomerulosclerosis may give a weakly positive reaction with Congo red. We have obtained slight reddish-brown staining of these lesions with Congo red after prolonged staining. However, with normal staining time, adequate to produce deep staining of islet and other amyloids, the renal lesions were negative. Furthermore, renal lesions faintly stained after prolonged immersion in Congo red were not birefringent or dichroic in polarized light.

sites of localized amyloidosis include the respiratory tract, genitourinary tract, bone and other organs.[35,36] Some of these may represent regional deposits in unrecognized systemic disease.[35]

There was no statistical correlation between the degree of sclerosis of pancreatic arteries and arterioles and the occurrence of islet amyloid. We encountered instances with very marked pancreatic arteriolosclerosis without amyloid in the islets and, on the other hand, very striking islet amyloidosis in pancreases with minimal arterio- and arteriolosclerosis. These observations are in agreement with those of Bell[11] and differ from those reported by Moschcowitz.[37] Similarly, there was no statistical correlation between islet amyloidosis and diabetic glomerulosclerosis in our series; this confirms previous reports.[11,38]

Our data regarding the incidence of amyloidosis of the islets of Langerhans in diabetic patients as compared with those not known to be diabetic are in essential agreement with those of Bell.[5] It should be emphasized that in none of our cases with amyloidosis of the islets in patients apparently free of diabetes had diabetes mellitus been ruled out by glucose tolerance tests. Islet amyloid is associated principally with the diabetes of maturity.[11] It is rare in juvenile diabetes[7,11] and is apparently absent in the diabetes of hemochromatosis.[39]

The pathogenesis of localized amyloid is not known. Abnormalities in the protein composition of blood serum, such as are known to be associated with systemic amyloidosis, have not been demonstrated in the circumscribed form, nor can anything be stated at present concerning the possibility of local disturbance in the protein milieu of the affected area. There seems little doubt that amyloidosis of the islets of Langerhans is in some way related to the diabetic state and does not simply represent a form of age change in an endocrine organ. Rinehart, Toreson and Abul-Haj[20] suggested that the hyalin might constitute a barrier to the release of insulin. It is also possible that it may be a consequence of some alteration in islet function. Nevertheless, the clearly established statistical relationship of diabetes mellitus and amyloid deposits in the islets of Langerhans points to the need for additional investigation of a possible pathogenetic linkage.

Summary

1. Hyalinization of the islets of Langerhans was found in 45 of a series of 91 consecutive necropsies in diabetic individuals over 50 years of age (49.5 per cent) and in 7 of 178 consecutive necropsies in individuals over 60 years of age not known to be diabetic (3.9 per cent).

2. Metachromasia with crystal violet and binding of Congo red were found in each instance of hyalinized islets. The Congo red-stained de-

posit exhibited dichroic birefringence in polarized light. These results, together with the general staining profile, permitted the identification of the hyaline substance as amyloid. Amyloid could not be identified in diabetic renal or vascular lesions.

3. Amyloidosis of the islets is a form of localized amyloid and occurs in approximately 50 per cent of diabetic patients over the age of 50 years.

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[*Illustrations follow*]

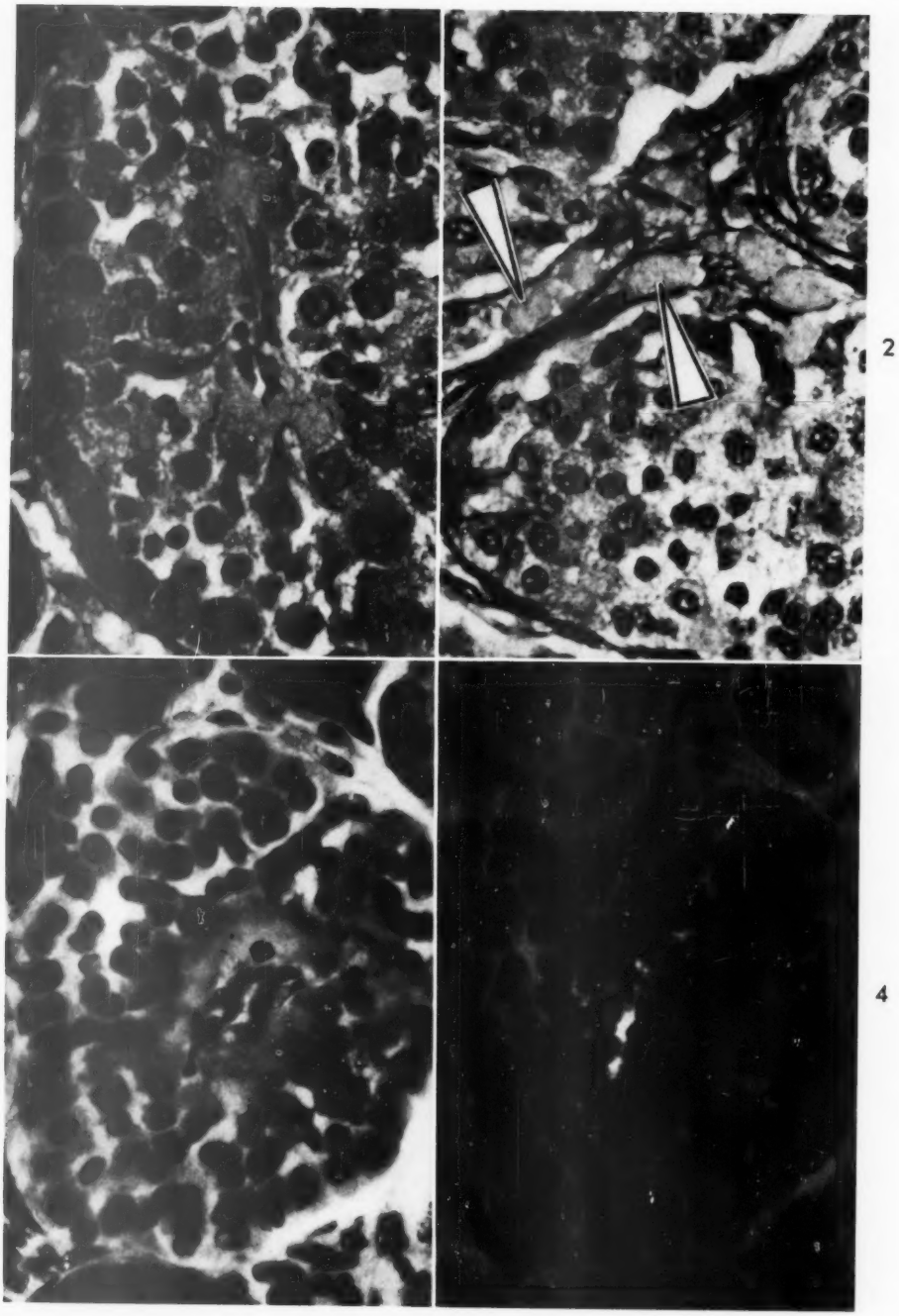
Legends for Figures

Fig. 1. Portion of an islet of Langerhans. Typical appearance of early or slight "hyaline" change. A pericapillary deposit of homogeneous material is located between the wall of a centrally located capillary and the islet cells. Hematoxylin and eosin stain. × 650.

Fig. 2. Portion of an islet of Langerhans. The islet capillary is at the upper right. Homogeneous deposits appear in the interstices of an argyrophilic fiber network (arrows). Silver and hematoxylin stain. × 650.

Fig. 3. Islet of Langerhans. The centrally located islet capillary is surrounded by a collar of homogeneous, strongly orange-red staining material. A few calcific particles are visible in the hyalin. Frozen section stained with Congo red-hematoxylin. × 650.

Fig. 4. The islet shown in Figure 3, viewed with polarized light. One strong and several weak anisotropic bands appear in the homogeneous deposit. Birefringence was dichroic; the bright band, for example, was green in one plane and yellow-pink after rotation through an angle of 90°. Anisotropism could not be demonstrated in this deposit prior to Congo red staining. × 650.



AMYLOID DISEASE OF THE BONE MARROW

Diagnosis by Sternal Marrow Aspiration

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Involvement of the bone marrow in generalized amyloidosis is thought to be relatively uncommon and is rarely recognized prior to necropsy examination. The diagnosis of amyloid disease of the marrow can be made by aspiration biopsy; however, in marrow films amyloid may be dismissed as technical artifact or nonspecific precipitated protein.

In the past 4 years we have encountered 3 cases of primary amyloidosis in which amyloid was found in films and sections of marrow obtained by aspiration biopsy. In one of these patients, amyloidosis was unsuspected until the substance was recognized in the marrow biopsy specimen. These cases prompted a review of marrow specimens previously aspirated from patients with amyloidosis and an examination of necropsy marrow specimens from cases of amyloidosis.

Clinical Observations

The 3 cases of primary amyloidosis included a 60-year-old woman with the nephrotic syndrome due to renal amyloidosis; a 56-year-old man with progressive dyspnea, edema, hepatomegaly, diarrhea and a bleeding tendency; and a 53-year-old man with a severe hemorrhagic diathesis due to a circulating fibrinolysin. In the first case the diagnosis of amyloidosis was established initially by renal biopsy. In the second case the diagnosis, made first by marrow biopsy, was confirmed by a liver biopsy. In the third case the diagnosis rested solely on the marrow alterations since the hemorrhagic tendency precluded biopsy of any other tissue. The circulating fibrinolysin, which was not due to prostatic carcinoma, was associated with fibrinogenopenia and cryoglobulinemia.*

In these 3 cases amyloid was recognized in marrow films stained with Wright's stain and in marrow sections stained with hematoxylin and eosin. The crystal violet stain was used for confirmation. The quantitative data and cell counts on the marrow films were not markedly ab-

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* This case was diagnosed by Dr. Lucille J. Hoilund. The fibrinolysin was demonstrated by Miss Lorraine Gonyea.

normal; however, there was a consistent increase in plasma cells which ranged from 2.2 to 5.2 per cent of nucleated marrow cells (normal, 1 per cent).

A review of marrow biopsy specimens previously obtained from patients with known or suspected amyloidosis revealed amyloid which had been unrecognized initially in specimens from 2 patients. In both cases marrow films exhibited no amyloid, but in sections this was found in the walls of the medullary vessels. One of these patients, a 63-year-old man with severe rheumatoid arthritis, had 3 marrow biopsy examinations, and amyloid was found in 2 of these. The 3 specimens, procured over a 2-year period, showed progressive hypoplasia of hematopoietic tissue and a progressive increase in plasma cells from 1.6 to 6.0 to 13.8 per cent. In one of the sections a lymphoid aggregate surrounding an amyloid-laden vessel whose lumen had been almost obliterated had been interpreted originally as an unusual type of granuloma presumably related to rheumatoid arthritis. On review, the true nature of this lesion became apparent through the use of special staining techniques. The other patient, a 69-year-old woman, had primary amyloidosis with macroglossia, amyloid goiter, left ventricular failure, and renal insufficiency. Sections of bone marrow showed only small amounts of amyloid in the medullary vessels, although at necropsy extensive deposits were found in the thyroid, kidney, pancreas, adrenal, heart, liver and spleen. Plasma cells comprised 5.2 per cent of nucleated marrow cells.

Necropsy Observations

Seventeen examples of amyloidosis were observed at necropsy at the University of Minnesota Hospitals between 1949 and 1959; however, in only 9 cases was bone marrow obtained for microscopic examination. These included 5 cases of primary amyloidosis and 4 of secondary amyloidosis (Table I).

The 4 cases of secondary amyloidosis exhibited a remarkably similar appearance of localized amyloid deposits in the marrow vessels. The marrow cellularity ranged from "markedly hypoplastic" to "slightly hyperplastic" in spite of the uniform degree of vascular amyloidosis. The cases of primary amyloidosis included one with massive marrow amyloidosis, 3 with localized deposits of amyloid in the marrow vessels and one with no demonstrable amyloid. The amyloid in the latter patient was found only in the myocardium.

Marrow aspirations had been done on 3 patients in whom necropsy marrow sections were available. In all of these, slight to moderate deposits of amyloid were found in the marrow vessels at necropsy, but

TABLE I

NECROPSIED CASES OF AMYLOIDOSIS, UNIVERSITY OF MINNESOTA HOSPITALS, 1949 TO 1959

Patient no.	Age	Sex	Diagnosis	Marrow biopsy	Marrow at necropsy	Necropsy observations
6	57	F	Primary amyloidosis		Massive amyloid replacement of marrow	Amyloid in pituitary, heart, small intestine, liver, spleen, pancreas, adrenal, kidney, cervix and lymph node
7	68	M	Primary amyloidosis	Plasmocytosis 3.8% No amyloid	Amyloid in vessels	Amyloid in liver, spleen and kidney
8	71	M	Primary amyloidosis		Amyloid in vessels	Amyloid in heart, intestine and skeletal muscle
9	45	M	Primary amyloidosis		Amyloid in vessels	Amyloid in thyroid, liver, heart and kidney
10	86	M	Primary amyloidosis		Normal marrow	Amyloid in heart
11	68	M	Rheumatoid arthritis; secondary amyloidosis		Amyloid in vessels	Amyloid in lung, liver, spleen, kidney, small intestine, adrenal, pancreas, prostate and bladder
12	21	M	Still's disease; secondary amyloidosis		Amyloid in vessels	Amyloid in liver, spleen, kidney, thyroid, adrenal, small intestine, heart and bladder
13	64	M	Rheumatoid arthritis; secondary amyloidosis	Plasmocytosis 1.2% No amyloid	Amyloid in vessels	Amyloid in pituitary, liver, spleen, adrenal and kidney
14	51	F	Tuberculous spondylitis; secondary amyloidosis	Plasmocytosis 1.4% No amyloid	Amyloid in vessels	Amyloid in kidney, liver and spleen

none was found in the biopsy specimens. The tissue obtained for sectioning was small in all of these cases and did not include small vessels which contained the amyloid at necropsy.

Identification of Amyloid in Marrow Films and Sections

In marrow films stained with Wright's stain, amyloid appeared as a structureless, homogeneous, purple-to-pink staining waxy material occurring in masses or clouds among the marrow cells (Fig. 1). On occasion it was sufficiently abundant to be regarded as a significant technical artifact or was present in streaks which, upon cursory examination, resembled aggregates of platelets. The masses of amyloid were either discrete or occurred in association with clusters of cells, fat or fragments of vessels. Isolated masses of amyloid gave a distinctive cumulus cloudlike effect, appearing light and transparent near the edges and more dense and billowy near the center. At the feather edge of marrow films or in preparations of the fat and perivascular cell layer of centrifuged marrow, the cloudy amorphous amyloid could often be seen in close proximity to the basement membrane of the capillary endothelium and the perivascular plasma cells. The differentiation of amyloid from nonspecific precipitated protein presented little difficulty; however, small accumulations of amyloid could mimic the cytoplasm of a megakaryocyte with its nucleus displaced through rupture of the cell membrane.

Some marrow sections showed large accumulations of amyloid displacing hematopoietic tissue (Fig. 2); in others, the amyloid was localized to the medullary vessels (Fig. 3). In those instances in which amyloid was limited to the vessels, the diagnosis, of course, depended upon detecting the affected vessels. The likelihood of finding these apparently varied with the amount of particulate tissue aspirated.

In the cases of primary amyloidosis, amyloid showed varying degrees of metachromasia with crystal violet from area to area in the same preparation and from patient to patient. All of the amyloid was strongly positive with the periodic acid-Schiff (PAS) stain, and this stain was useful for more detailed morphologic investigations. To avoid bias in observations on PAS-stained tissue, serial sections were stained with crystal violet and PAS, and all observations were confirmed in the crystal violet-stained sections. The homogeneous appearance and the size of the deposits distinguished amyloid from clusters of platelets and the cytoplasm of megakaryocytes, but special staining characteristics were, necessarily, not helpful in this differentiation since amyloid, platelets and megakaryocytes all had similar reactions to the crystal violet and PAS stains. A useful procedure was treatment of the tissue with

ptyalin, which resulted in a much weaker reaction to the PAS stain in platelets and megakaryocytes while the reaction of amyloid remained unchanged.

Development of Amyloid Disease of the Bone Marrow

By utilizing both biopsy and necropsy tissue, it was possible to demonstrate all stages of marrow amyloidosis from minimal vessel involvement to massive replacement of the marrow parenchyma. In our series the latter occurred in primary amyloidosis only, while localized vessel involvement was found in both primary and secondary amyloidosis. It was not possible to distinguish primary from secondary amyloidosis morphologically if both had similar degrees of marrow involvement.

The earliest stage of marrow amyloidosis appeared to be characterized by localized deposits in the medullary vessels. These ranged from minimal accumulations in the media, which could be identified only with amyloid stains, to large amounts narrowing the vascular lumens and greatly thickening the walls. With more pronounced involvement, small clumps of amyloid could be seen spreading off into the perivascular connective tissue along the reticular fibrils and dispersed in the marrow substance. The isolated masses of amyloid appeared to coalesce into larger aggregates which contained normal-appearing hematopoietic cells within their interstices. At this stage blood vessels contained large amounts of amyloid, and in some areas this appeared contiguous with the extramural deposits (Fig. 2). Even with extensive amyloidosis the distribution in the marrow was spotty. Some portions contained only normal hematopoietic tissue, while adjacent areas showed almost complete replacement by amyloid.

A consistent finding in the marrow in both primary and secondary amyloidosis was a slight to moderate increase in plasma cells. There was moderate variation in size and structure of these cells, but in no case did they show the usual characteristics of myeloma cells. With the crystal violet stain some of the plasma cells exhibited a diffuse cytoplasmic metachromasia. Near vessels some plasma cells were almost completely embedded in amyloid, and many isolated cells had a cloudy film of this substance at the periphery of the cytoplasm.

Russell bodies were found in plasma cells in both primary and secondary amyloidosis (Fig. 4), and in sections the sequence of their formation could be traced. Initially, the cytoplasm stained diffusely with the PAS stain; later PAS-positive cytoplasmic granules appeared, and these in turn coalesced to form the brilliantly PAS-positive Russell bodies. Some plasma cells were only mildly PAS-positive, and their

cytoplasm was filled with clear vacuoles. In some the vacuoles were lined by a brilliantly PAS-positive deposit, apparently the remnants of Russell bodies. Many plasma cells adjoining amyloid deposits showed no Russell body formation, and others containing Russell bodies could be found unassociated with amyloid deposits. The sequence of Russell body formation in crystal violet-stained preparations paralleled that in the PAS stain.

Discussion

The first observation of marrow involvement in amyloidosis was made in 1872 by Ponfick[1] who noted deposits in the small and medium-sized medullary arteries. This was 19 years after the term "amyloid" was first used by Virchow and several decades later than the first descriptions of visceral alterations compatible with amyloidosis.[2] The first report of extensive marrow involvement, comparable to that seen in the liver and spleen, was made by Gerber in 1934.[3] At necropsy this patient's vertebrae, ribs and ilia exhibited almost complete replacement of the marrow by a putty-like substance.

Since Gerber's paper, additional examples of primary amyloidosis with extensive marrow involvement have been published. In one case a pathologic fracture of the femur was attributed to amyloid deposits.[4] The apparently low incidence of marrow involvement in this disorder has been puzzling and was attributed by Magnus-Levy[5] to the enclosed circulation found in the marrow.

Reported data on the incidence of marrow involvement may not be reliable because the marrow is not always examined at necropsy. Moreover, minimal vessel involvement may be missed without special stains for amyloid. Mathews[6] reported bone involvement in 10 cases in a series of 98 collected up to 1954. Rukavina and colleagues[7] reviewed 154 cases and found that of 61 cases in which observations on the marrow were recorded at necropsy, 11 (18 per cent) had amyloid in the marrow. Sixteen of Rukavina's cases had examination of marrow aspirations prior to death, and all were reported negative for amyloid and myeloma.

Although our series is too small for statistical evaluation, it suggests that the incidence of marrow involvement may be greater than previous reports have indicated since marrow amyloidosis was found in 8 of 9 cases (89 per cent) in which necropsy sections were available. In 7 of these, marrow amyloid was limited to the medullary vessels.

Amyloid disease of the marrow apparently is rarely recognized before necropsy. Reported instances in which amyloid was found in films of aspirated marrow include a case of multiple myeloma reported by

Trubowitz,[8] one of primary amyloidosis mentioned by Lee, Michael and Vural[9] and two unusual examples associated with plasmacytosis and nonthrombocytopenic purpura reported by Propp, Scharfman, Beebe and Wright.[10] Two other cases in which amyloid was found in marrow particles obtained by aspiration have been reported, but apparently the marrow films did not exhibit the substance; the extent of involvement was not described.[11,12]

Amyloid deposit localized to the medullary vessels appears to be the most common form of this disorder, and this represents the earliest stage at which the amyloid can be identified for diagnostic purposes. Descriptions of the progression of the lesion have not been encountered in published reports, and there is disagreement concerning the initial site of amyloid deposition in other tissues.[13]

The association of plasmacytosis and amyloidosis has been observed frequently.[2,5–8,10,14] It appears likely that plasma cells are the source of the material which ultimately is deposited as amyloid. Intracytoplasmic amyloid has been reported in plasma cells and has been interpreted as indicating intracytoplasmic synthesis.[11,14] This was based on the assumption that plasma cells are not phagocytic. However, it should be pointed out that plasma cells may contain bacteria, erythrocytes, hemosiderin and various crystals; [15] thus they or their precursors may well have phagocytic potentialities.

Russell bodies have been reported in the marrow in amyloidosis secondary to multiple myeloma [16] and in primary amyloidosis.[17] They also occur in numerous conditions in which marrow plasmacytosis is unassociated with amyloidosis.[18] Our observations of the development of these bodies are in agreement with those of Pearse.[19] Both confirm Downey's early impressions [20] that "Russell bodies are the expression of a final secretory act in the life history of the plasma cell." It is clear that they are not unique to amyloidosis, but their relationship to the formation of amyloid remains an intriguing possibility.

The ante-mortem recognition of amyloidosis rests upon the demonstration of the substance in a biopsy specimen. Biopsy examination of some tissue other than the marrow is more likely to give diagnostic results. On the other hand, in those cases where a hemorrhagic diathesis precludes needle puncture of the liver or kidney, aspiration biopsy of the marrow represents a safe substitute procedure. Blood loss may then be recognized and measures taken to arrest it. More important than this, however, is the fact that amyloidosis may be recognized during routine examination of marrow specimens taken for other purposes. This can be done only if the examiner is aware of the appearance of amyloid in marrow films and is alert to the diagnostic possibilities.

Summary

1. The diagnosis of amyloid disease can be made by aspiration biopsy of bone marrow. Three cases of primary amyloidosis in which the deposit was demonstrated in films and sections of marrow obtained by sternal aspiration are cited.

2. A survey of marrow biopsy specimens obtained previously from patients with known or suspected amyloidosis revealed that amyloid had not been recognized in two instances.

3. Amyloid was found in the marrow in 8 of 9 cases of amyloidosis examined at necropsy. In 7 of these there was limited vessel involvement, and in one the marrow was extensively replaced.

4. A consistent observation was a slight to moderate marrow plasmacytosis in both primary and secondary amyloidosis. Russell bodies appeared in plasma cells in both forms of the disorder, and the sequence of their formation could be traced.

5. The amyloid deposit first appeared in the small vessels of the medullary cavity and then spread into adjacent hematopoietic tissue, finally replacing much of the marrow.

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[Illustrations follow]

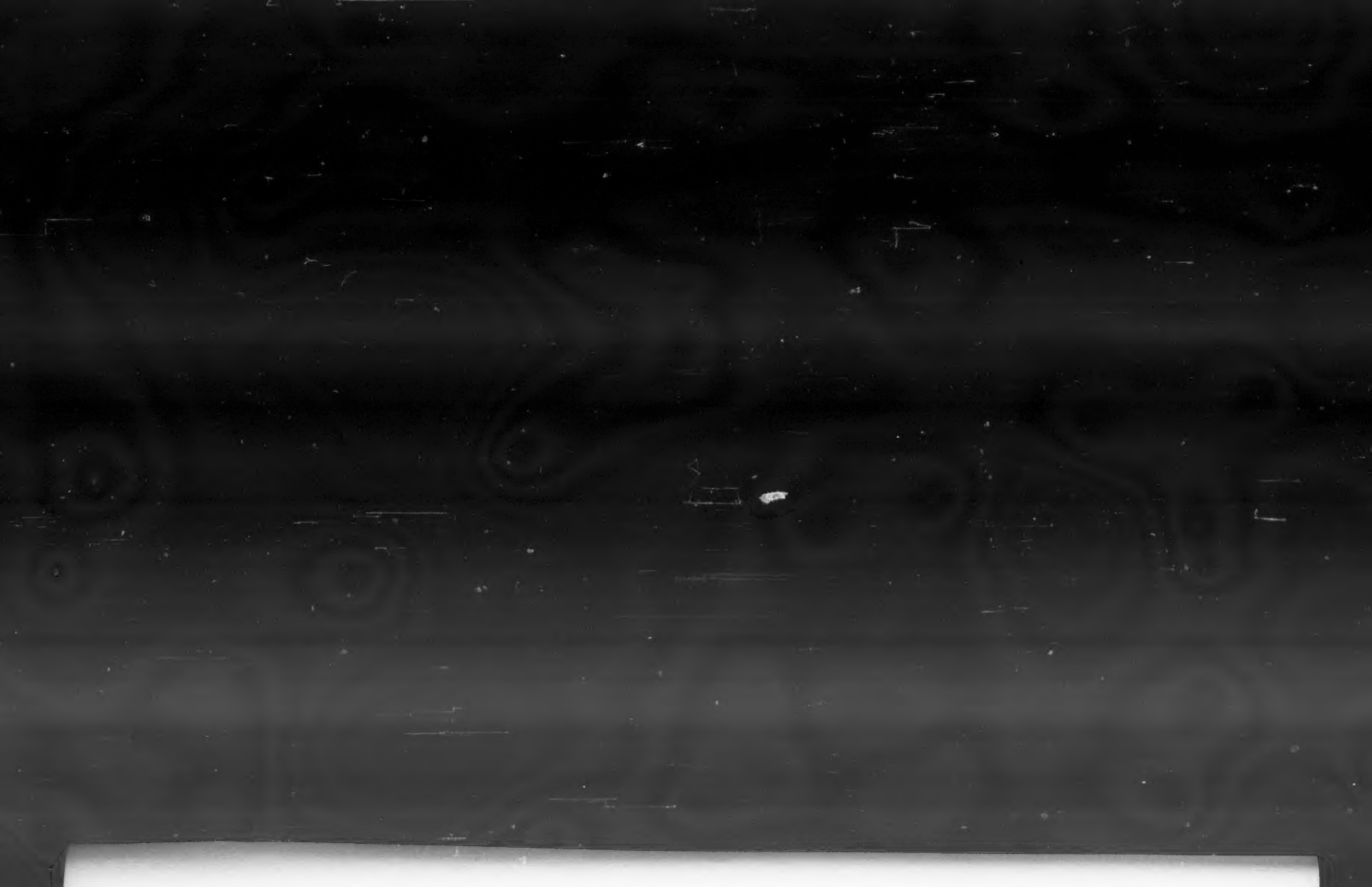
Legends for Figures

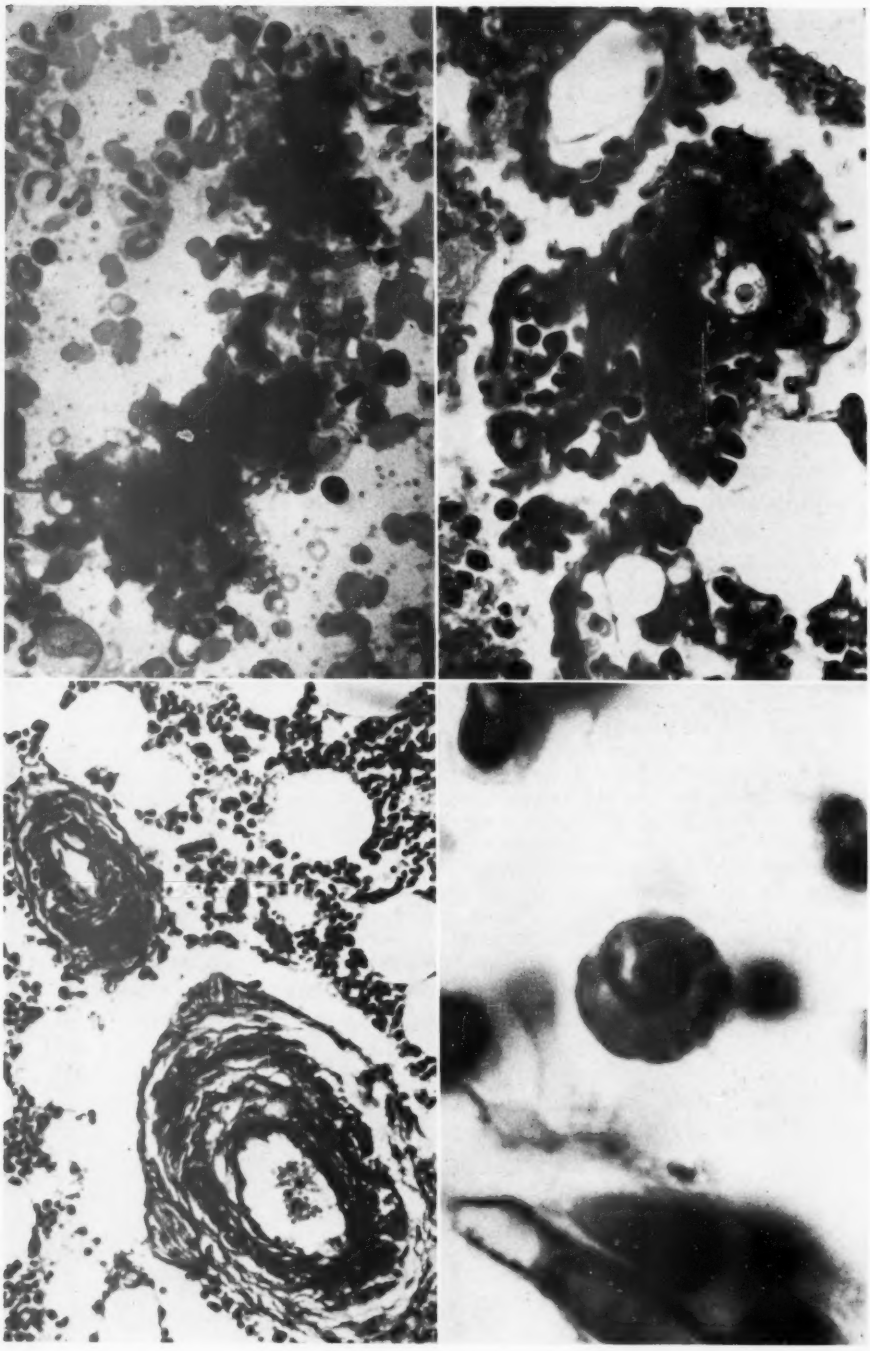
Fig. 1. Case 1, primary amyloidosis. Large amorphous masses of amyloid are scattered among marrow cells. Marrow film, Wright stain. × 1400.

Fig. 2. Case 1, primary amyloidosis. Section of marrow particles obtained by aspiration. A small marrow vessel is surrounded by darker staining amyloid which is contiguous with parenchymal deposits of similar substance. Periodic acid-Schiff (PAS) stain. × 480.

Fig. 3. Case 11, secondary amyloidosis. Necropsy marrow specimen. Small vessels contain a moderate deposit of amyloid in the media. PAS stain. × 220.

Fig. 4. Case 14, secondary amyloidosis. Necropsy marrow specimen. Large globular Russell bodies are seen in the cytoplasm of a plasma cell. PAS stain. × 1600.





THE EARLY PENETRATION OF EXPERIMENTAL GASTRIC ULCERS IN RATS RECEIVING CORTISONE

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Although the pathogenesis of human peptic ulcer is not well understood, one of its important features is its tendency to penetrate or perforate, complications which are particularly common in patients receiving cortisone.[1] Skoryna, Webster and Kahn[2] have reported that in rats receiving cortisone, experimental gastric ulcers increased in size and showed a high incidence of both penetration and perforation. However, in that study the animals were not examined until the third day after the production of the ulcer; by this time deep penetration had already occurred. The present investigation is concerned with the early effects of cortisone administration on experimental gastric ulcer in the rat.

MATERIAL AND METHOD

Male rats of the Royal Victoria Hospital strain, weighing 180 to 200 gm., were maintained on Purina meal diet and water *ad libitum*. By a method previously described,[3] a standard ulcer, 6 mm. in diameter, was produced on the posterior wall of the glandular part of the stomach with thermocautery. After the ulcers had been induced, the rats were divided at random into 3 groups: Group I, 60 rats with experimental ulcer; no cortisone was administered. Group II, 60 rats with experimental ulcer; cortisone acetate (supplied through the courtesy of Merck, Sharp & Dohme Company, Ltd., Canada), 0.025 mg. per gm. of body weight, was administered intramuscularly daily beginning on the day the ulcer was induced. Group III, 60 rats with experimental ulcer; cortisone acetate, 0.075 mg. per gm. of body weight, was administered intramuscularly daily beginning on the day the ulcer was induced.

Ten animals of each group were sacrificed 6, 12, 24, 48 and 96 hours and 7 days after production of the ulcer. The stomachs were fixed in formalin, and sections were stained with hematoxylin and eosin or by Masson's trichrome method.

RESULTS

Mortality

The only mortality occurred in rats receiving the higher cortisone dosage (group III) which survived beyond 96 hours. The animals in this group became very weak and 5 died spontaneously between 96 hours and 7 days.

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Weight

Animals not receiving cortisone (group I) regained their pre-operative weight in 96 hours and continued to gain weight for the remainder of the 7-day period. Animals receiving the lower cortisone dosage (group II) lost 15 to 20 gm. during the first 4 days, but thereafter their weight remained stationary. Rats on the higher cortisone dosage (group III) lost 15 to 20 gm. during the first 96 hours, and another 10 to 15 gm. in the subsequent 3 days.

Gross Observations

Group I (no cortisone). At 6 and 12 hours after operation, the ulcers were 6 mm. in diameter and were slightly depressed. Neighboring organs frequently formed support for the ulcer site, but adhesions were not found until 48 hours. At this time there was an inflammatory reaction over the serosa, and adhesions with the underlying liver, spleen or other regional structures were formed. At 96 hours the ulcers averaged 7 mm. in diameter. They had a punched-out appearance with raised edges, and the floor was covered by necrotic material. Penetration through the entire thickness of the gastric wall did not occur; thus, though the neighboring structures were loosely adherent to the base, they never formed the actual floor of the ulcer. By the seventh day the ulcer site was thicker than the surrounding normal gastric wall, and the ulcer floor and base consisted of a layer of firm gray tissue. There was no penetration into the neighboring organs.

Group II (cortisone, lower dosage). In the first 24 hours, the ulcers of this group resembled those of the control group closely except that they were slightly larger, the average diameter at 24 hours being 8 mm. Subsequently the ulcers were always larger than in the control group, and by 96 hours all of them had penetrated into adjacent viscera. In some, the adherent organ could be seen in the ulcer floor as if closing a perforation. At 7 days, penetration into the adherent organ was still apparent, but a layer of firm gray tissue now comprised the ulcer wall.

Group III (cortisone, higher dosage). At 24 hours, the ulcers in this group were twice as large as those in the control group, measuring, on the average, 12 mm. in diameter. All had penetrated into organs adherent to their bases. By 96 hours, the ulcers had extended laterally, and many of them measured as much as 15 mm. in diameter. They penetrated deeply into adjacent organs so that they formed a deep cul-de-sac, the base of which consisted of liver, pancreas or spleen. At 7 days the ulcers appeared much as they had at 96 hours. The layer of firm gray tissue found in the ulcer floor in the other groups was not apparent.

Histologic Observations

Six to 12 Hours. There was no histologic difference between the cortisone-treated and the control groups. The ulcer site showed a wide zone of coagulative necrosis which involved the mucosa, submucosa, muscularis, and in many instances reached the serosa. The general architecture of the gastric wall in this area was distorted, shrunken, and cytologic detail was lost. A marked hyperemia with exudation of edema fluid, fibrin and a few leukocytes was present in the submucosa and subserosa of the unburned tissue at the margin of the ulcer.

Twenty-four hours (Figs. 1 to 3). In group I (no cortisone) the general architecture of the ulcers was much as it was at 12 hours. There was, however, an increase in the inflammatory response with more leukocytic exudation; minor penetration of the burned tissue by neutrophils was initiated. Submucosal edema had increased so that the surrounding intact mucosa was slightly raised. A fibrinopurulent exudate extended over the serosa, and there were adhesions to neighboring organs which formed support for the base of the ulcer.

The ulcers were larger in group II (cortisone, lower dosage), but otherwise resembled those in group I.

In group III (cortisone, higher dosage) all the ulcers were larger and deeper than in the group not receiving cortisone. They penetrated deeply, in many cases destroying the entire thickness of the gastric wall and extending into an adherent neighboring organ (pancreas, spleen, liver or small intestine). Thus the floor of the ulcer consisted of the necrotic tissue of this adherent organ. There was a marked acute inflammatory exudate surrounding the necrotic area.

Forty-eight Hours. The ulcers of the animals in group I (no cortisone) now extended almost to the serosa, their floors consisting of the necrotic residue of the burned wall. Although organs were adherent to the serosa, no ulcer had penetrated into these, the serosa remaining intact in every case. A more pronounced inflammatory exudate and early but definite fibroblastic proliferation was present at the base and edges of the ulcer. The viable mucosa at the ulcer margins was raised by submucosal edema; its epithelium showed mitotic activity, and a single row of epithelial cells extended from the periphery for a short distance over the necrotic surface.

The ulcers in the animals receiving the lower dosage of cortisone (group II) were similar to those of the control group in regard to inflammatory response, fibroblastic proliferation and epithelial regeneration. They were, however, larger and penetrated more deeply. In some cases they penetrated the serosa and extended a short way into a neigh-

boring organ. The ulcer edge was undermined, and the viable mucosa, including its muscularis mucosae, was raised and thrown into a large fold.

In group III (cortisone, higher dosage) the ulcers were larger and more deeply penetrating than those at 24 hours, and were much larger than those in the other groups at 24 or 48 hours. They extended through the gastric wall into a neighboring adherent viscus, and large areas of necrosis were present in the adherent organ. In the regions of acute necrosis, no granulation tissue was evident; in adjacent areas, however, fibroplasia was present and was of the same degree as in the control group. The undermining and folding of the mucosa at the ulcer edges in group II was even more prominent here. The inflammatory response and epithelial regeneration were similar to the control group.

Ninety-six Hours (Figs. 4 to 6). The ulcers in group I (no cortisone) were larger than at earlier times but had not penetrated the serosa. The ulcer floor was covered by a fibrinopurulent layer; deep to this was a wide band of granulation tissue which made up the ulcer wall and base. Epithelial regeneration had progressed.

In group II (cortisone, lower dosage) the ulcers were now much larger than in the control group. Many had penetrated deeply into adherent organs; the appearance was similar to that seen at 24 hours in group III. The ulcer floor consisted of a necrotic zone in the adherent organ. In all cases a band of granulation tissue originating in the penetrated organ formed the major portion of the ulcer wall and base, though the amount of granulation tissue was less than in the control group. The undermining and mucosal folding at the ulcer edges continued to be prominent. There were necrosis and an inflammatory exudate in the submucosa in this region. Epithelial regeneration was similar to the control group.

Group III (cortisone, higher dosage) continued to show the most marked penetration. The ulcers were large and deep. Wide areas of recent necrosis continued to be manifest in the organs forming the ulcer floor and at the ulcer margins. In some, necrosis was so extensive that the ulceration appeared to be on the point of perforating through the entire thickness of the adherent organ. There was some granulation tissue in the penetrated organs, but this was patchy in distribution and was much thinner than in either of the other two groups. The peripheral undermining was very marked, and necrotic tissue extended for a distance deep to the adjacent intact mucosa. Because of the raised mucosal fold which overhung the ulcers, epithelial regeneration was difficult to evaluate; however, in general, it appeared to be of the same degree as in the control group.

Seven Days (Figs. 7 to 9). The floor of the ulcers in group I consisted of a thin layer of necrotic debris. The wall was now markedly thickened by a wide zone of vascular granulation tissue. A small number of collagen fibers were present at the base and edges. Epithelial regeneration had continued, and the row of epithelial cells over the surface was longer.

In group II the ulcers were of approximately the same size as at 96 hours. Though larger and more deeply penetrating than in the control group, they were similar to these with respect to granulation tissue and epithelial regeneration.

The large ulcers in group III were of approximately the same size as at 96 hours. Areas of acute necrosis accompanied by inflammatory exudate were still present in the penetrated organs and at the peripheral undermined margins. In many instances colonies of bacteria and fungi could be identified in the floor. A discontinuous band of granulation tissue was present at the ulcer base and edges, but in some areas collagen fibers could be identified. The amount of granulation tissue was much less than that seen in either of the other groups.

Discussion

The present investigation clearly indicated that cortisone administration in the rat resulted in an early increase in the size and depth of penetration of the experimentally induced gastric ulcer. The ulcers would undoubtedly have perforated except that organs adjacent to the stomach were freely movable and readily migrated to areas of serosal inflammation. The observations also confirmed our previous suggestion [2] that in this type of gastric ulcer the delay in healing caused by cortisone administration was attributable primarily to a larger area of ulceration and the need to repair a greater degree of penetration. The "delay" was related to the size of the defect rather than to the rate of repair.

The results obtained also indicated a relationship between the cortisone dosage and the onset and degree of penetration. With the higher dose of cortisone the ulcers were larger and had penetrated more deeply into adjacent organs by 24 hours and continued to increase in size and depth up to 96 hours. At 7 days the ulcers in this group still showed necrosis and acute inflammation, and the base contained only a thin, discontinuous layer of granulation tissue. With the lower dose of cortisone, penetration and enlargement were first apparent at 48 hours and progressed so that by 96 hours penetration into adherent organs had occurred. In the control rats the ulcers did not increase in size significantly and did not penetrate through the serosa.

The effect of cortisone on fibroplasia varied in the two cortisone

groups. At 96 hours after the induction of the ulcer, the amount of granulation tissue present was less in the group given the lower dose of cortisone than in the control group, but by 7 days the amount of granulation tissue was approximately the same. With the higher dose at both 96 hours and 7 days, only a small amount of granulation tissue was found. However, in another investigation we found granulation tissue to be abundant in rats given larger amounts of cortisone and examined 15 days after the induction of the ulcer. Cortisone did not appear to affect epithelial regeneration.

The method used produced a standard ulcer which involved almost the full thickness of the stomach wall. Other investigators reporting the effects of cortisone on experimental gastric ulcers have studied much more superficial ulcerations and have obtained rather different results. Williams,[3] Rodriguez-Olleros and Gallindo,[4] Myhre[5] and Janowitz and colleagues[6] found no retardation of gastric mucosal regeneration in animals receiving cortisone. Janowitz and co-workers[6] found some retardation of healing of excisional mucosal defects in the dog, and Myhre[7] observed delay in healing of sutured gastrotomy wounds in the fundus of the rat. In the latter two experiments it was suggested that the delay in healing was due to cortisone-induced depression of fibroplasia.

It is our suggestion that the factors having a potential role in penetration be divided into two groups: those that could enhance the spread of the ulceration, and those that could limit it. Factors that could enhance the ulcerative process include alterations in the gastric secretory activity or the mucin barrier, release of proteolytic enzymes, promotion of necrosis in the ulcer bed by infection or neurocirculatory disturbances and depression of the inflammatory response. The limiting factors include connective tissue repair, muscle contraction and changes in mucopolysaccharide composition in the ulcer bed.

Variations in gastric acidity or volume do not seem adequate to explain the greater size and penetration of the ulcers in the rats given cortisone. In the strain used in the present experiment no significant alteration in gastric secretory volume or acidity was produced by cortisone administration.[8] Robert and Nezamis[9] and Kyle and Welbourn[10] have also reported no significant cortisone-induced change in gastric acidity. Moreover, the administration of gastric secretory stimulants such as histamine or pitressin did not result in increased size or penetration of the experimental gastric ulcers though these drugs did cause an increase in the acidity and volume of the gastric juice.[11]

The effect of cortisone on gastric mucin in relation to the acid-mucin barrier[12] or to proteolytic enzymes could not be evaluated in relation to the results obtained in this experiment. The greater penetration did

not appear to be due to continued necrosis in the ulcer bed, secondary to either infection or neurocirculatory disturbance. Sidransky and Friedman [13] have shown that cortisone rendered mice highly susceptible to pulmonary aspergillosis; one of the well known actions of cortisone is its antiphlogistic effect. Bacteria and fungi were found in the ulcers in the present experiment. However, this was not manifest until after several days of cortisone therapy whereas deep penetration had occurred by 24 hours. Moreover, depression of the inflammatory response was not observed in the cortisone-treated animals in the present study.

Lambling and colleagues [14] suggested that focal vascular disturbances and thrombosis played principal roles in the pathogenesis of steroid-induced ulcers. No evidence was found in the present experiment to suggest that thrombosis had a primary role in early penetration. In addition, neither histamine nor pitressin had any effect on either the size or the penetration of the experimental ulcer [15] although both of these compounds have been reported to affect the blood supply to the stomach in some species.[16]

With regard to the factors which might limit ulcer penetration, the most commonly accepted is repair by fibrous tissue. Cortisone has been shown to depress fibrous tissue production if given in high enough dosage.[17] In experimental group III given the higher dose of cortisone, deep penetration had occurred by 24 hours after induction. This would appear to rule out any relationship to fibroplasia since significant fibroblastic proliferation does not normally occur within 24 hours after an injury. Indeed, despite the fact that fibroplasia was not significantly depressed, deep penetration still occurred in the lower dose cortisone group.

"Wound contraction," the approximation of wound margins without new tissue production, constitutes an important method of closure in skin wounds. Cortisone has been shown to delay the onset of "wound contraction" in the skin of the Wistar rat,[18] but this phenomenon plays no significant role in the normal healing of the experimental gastric ulcer we have investigated.[19] Ferguson [20] demonstrated that the size of wounds made by excising patches of gastric mucosa in the dog was considerably reduced by contracture of the underlying smooth muscle. Janowitz and co-workers [6] observed similar but temporary contraction in mucosal defects of canine gastric explants. We have not been able to determine exactly what part the muscle layer may play in limiting the spread of gastric ulceration in the rat. Cramer and Nadel [21] found the bursting strength of sutured gastrotomy wounds to be reduced at 24 hours in mice treated with prednisone as compared with untreated animals. The authors were unable to explain this, but its occurrence at such

an early stage of healing would seem to eliminate depressed fibroplasia as a factor. Cortisone has been shown to have some as yet undefined effect on striated muscle in animals [22] and human beings,[23] but its effect on smooth muscle has not been established.

Mucopolysaccharides might have some importance in the maintenance of stability of an injured gastric wall. Cortisone has a definite effect on mucopolysaccharide synthesis.[24] Asboe-Hansen [25] has suggested that mucopolysaccharide composition and the state of polymerization may be affected by this steroid. The present experiment provided no evidence in support of this hypothesis.

The relationship between the administration of cortisone and the development of peptic ulcer is controversial. The increased incidence of hemorrhage and perforation in ulcer patients on cortisone therapy seems well documented,[1] and these complications may be related to rapid penetration of the ulcer. Palmer and Kirsner,[26] however, have recently questioned the existence of a direct relationship between cortisone therapy and the development of peptic ulcer in human subjects. Aagaard, Andreassen and Schiødt [27] have reported 10 patients receiving long term steroid therapy in whom gastrectomy was performed for active peptic ulcer. The ulcers in this group did not differ from ordinary chronic penetrating peptic ulcers, and the fibrous tissue response was considered to be normal. The effect of cortisone on the gastric secretory mechanism in the human subject is disputable. Gray, Ramsay, Reifenstein and Benson [28] reported that it resulted in an increase of secretory volume, acid and pepsin, whereas Beck, Fletcher, McKenna and Griff [29] found no such increase.

Some aspects of the evidence obtained in studies of human patients are consistent with our own experimental observations in the rat. There is little doubt that cortisone administration resulted in extension and increased penetration of the type of experimental gastric ulcer investigated. This was most pronounced in the higher dosage range, but it also occurred with a much lower dose. For the reasons stated, it seems highly unlikely that either gastric secretory derangements or depression of fibroplasia played any significant role.

Summary

The early effects of cortisone administration on experimental gastric ulcers produced in rats by thermocautery are reported. Two levels of dosage of cortisone (0.025 mg. per gm. of body weight per day and 0.075 mg. per gm. per day) were used; controls received no cortisone. Animals were sacrificed 6, 12, 24, 48, 96 hours and 7 days after the induction of the ulcers. Extension and penetration of the ulcers were ob-

served in animals receiving cortisone; the effects were more pronounced with higher doses.

Factors which might play a role in the penetration were considered to be those that could enhance the spread of the ulceration and those that could limit its penetration.

It seemed most unlikely that the mechanism by which cortisone administration resulted in increased penetration was related to either gastric secretory derangement or fibroplasia. It also seemed improbable that neurovascular disturbances played a significant part.

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LEGENDS FOR FIGURES

Illustrations were prepared from sections stained with hematoxylin and eosin.

Figures 1 to 3 illustrate areas of ulceration 24 hours after induction. × 12.

FIG. 1. Group I, control, no cortisone. The ulcer has not penetrated through the serosa.

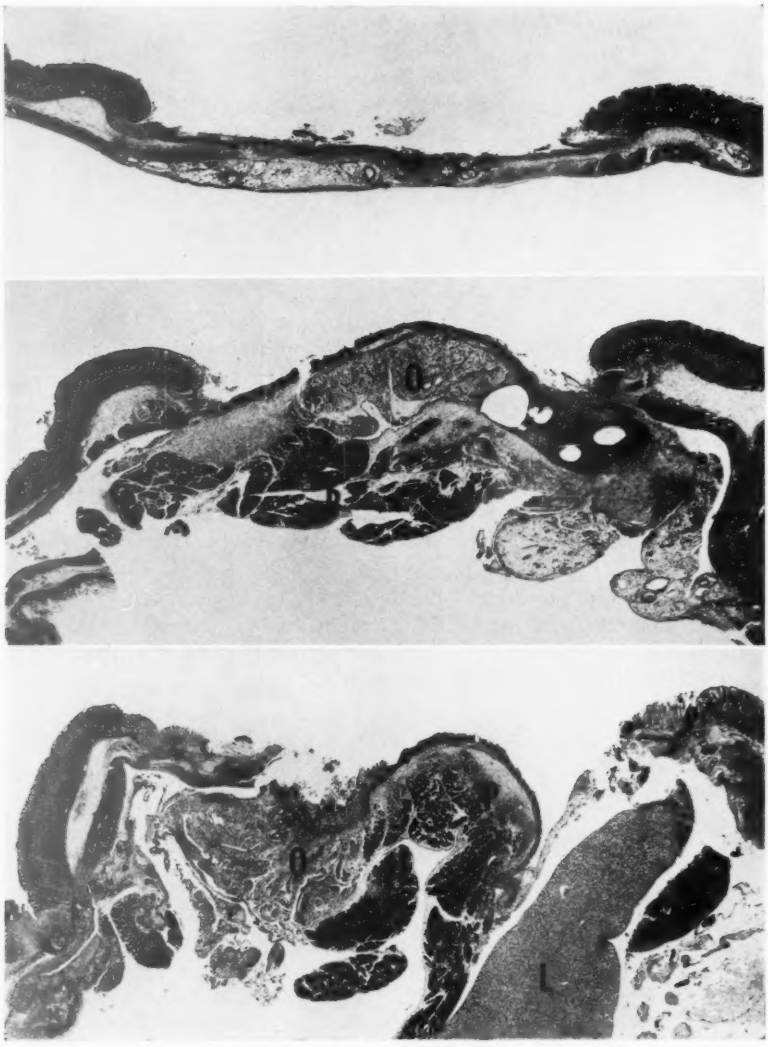

FIG. 2. Group II, cortisone, lower dose. The ulcer has penetrated just through the serosa into the adherent omentum (O) and pancreas (P).

FIG. 3. Group III, cortisone, higher dose. The ulcer has penetrated deeply into the adherent omentum (O), pancreas (P), and liver (L).

Figures 4 to 6 show areas of ulceration 96 hours after induction. × 12.

FIG. 4. Group I, control, no cortisone. The ulcer now resembles a healing peptic ulcer in the human subject. There is a broad band of regenerating fibrous tissue at its base. The pancreas (P) and the spleen (S) support the base but have not been penetrated to any extent.

FIG. 5. Group II, cortisone, lower dose. The ulcer is much larger and deeper than in the control. There is penetration of the liver (L) and pancreas (P). A band of granulation tissue arises from these organs but is less in amount than in the control.

FIG. 6. Group III, cortisone, higher dose. The ulcer is a very complex appearing lesion because of deep penetration into the liver (L) and pancreas (P). There is only a narrow discontinuous band of granulation tissue arising from the surface of the organs forming the base of the ulcer.

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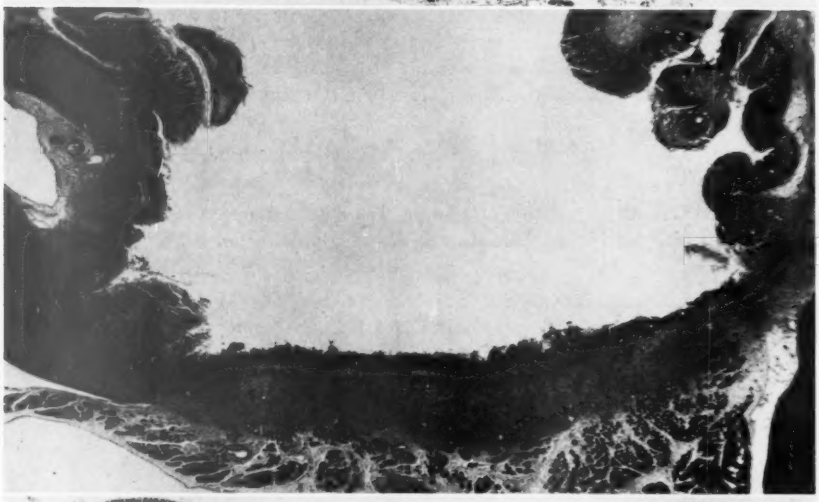

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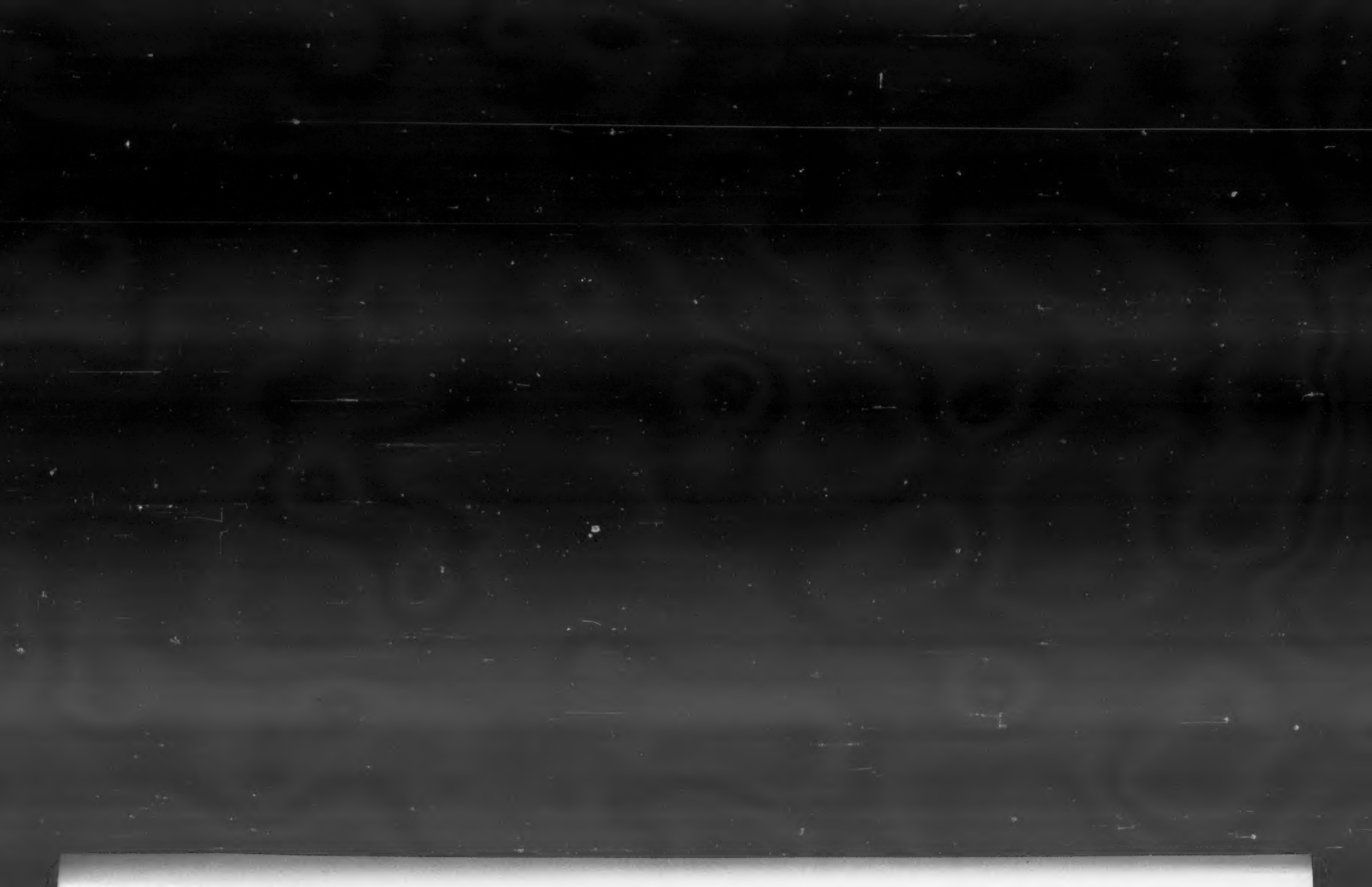
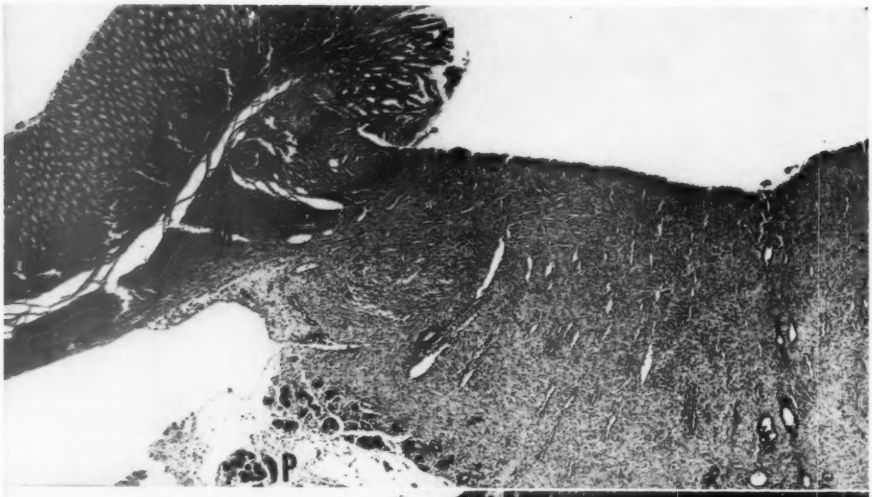
Figures 7 to 9 illustrate areas of ulceration 7 days after they were induced. The amount of granulation and connective tissue at the base of the ulcers in the 3 groups can be compared. There is normal gastric mucosa and muscle coat (M) at the edge of the ulcers and pancreas (P) and omentum (O) supporting or comprising their bases. × 40.

FIG. 7. Group I, control, no cortisone. There is a wide band of regenerating connective tissue in the base of this ulcer.

FIG. 8. Group II, cortisone, lower dose. The regenerating connective tissue is less in amount and less mature than that in the control ulcer.

FIG. 9. Group III, cortisone, higher dose. The amount of regenerating connective tissue in the ulcer base is much less in this group than in the other two groups. Extension of the ulcer by peripheral undermining with necrosis of the submucosa and muscle coat (M) is apparent.





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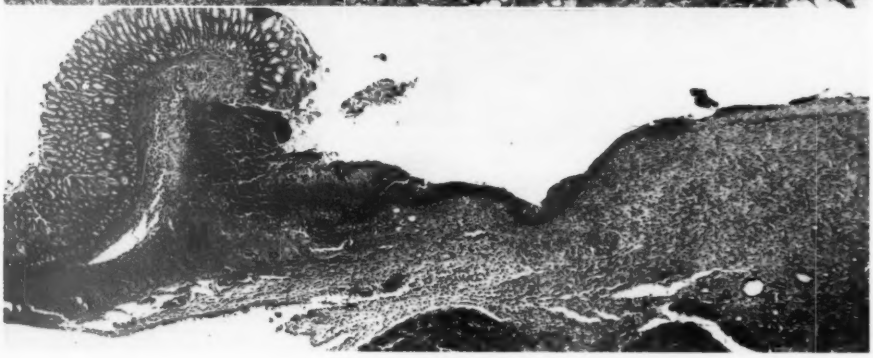

9

EXPERIMENTAL PULMONARY EMBOLISM WITH SERUM-INDUCED THROMBI

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Since Virchow demonstrated the relation between pulmonary artery obstruction and infarction of the lung, numerous techniques have been employed to duplicate experimentally the pathologic and physiologic events that follow embolization of the lesser circulation. The introduction into the venous blood of a variety of substances has not succeeded in reproducing fully the dynamic factors involved in the production of thrombi, their release, their passage to the lung, and the mechanisms by which they may be removed from the circulation.

To investigate some of these factors in a more physiologic and controlled setting than has heretofore been available, use was made of the observation that the systemic infusion of thrombin-free serum induced massive thrombosis in vascular segments containing stagnant blood far removed from the site of infusion.[1] This method of thrombus formation is simple and reproducible; the thrombi formed are initially nonadherent and of uniform composition, can be of predetermined size, and can be produced without significant intimal injury or systemic disturbance in the veins and arteries of a variety of animals.[2] The present report describes some of the observations resulting from an adaptation of this technique to the study of pulmonary embolism.

METHODS AND MATERIAL

The basic technique of thrombus formation has been described previously.[1] Forty mongrel dogs, 15 to 20 kg. in weight, were anesthetized with sodium pentobarbital, and a segment of each external jugular vein was freed from its surrounding structures, and its tributaries ligated. Thirty ml. of heterologous, thrombin-free, canine serum eluate,* independent of the weight of the animal, was then infused into an antecubital vein in 30 seconds. Within 60 seconds after completion of the infusion,

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* This eluate was prepared by treating pooled canine serum with barium sulfate, centrifugating and eluting the adsorbate with citrate, as previously reported in detail.[1] The eluate represented a 4-fold concentration by volume of the thrombosis-accelerating activity of the parent serum and was used in place of serum in these and subsequent experiments to reduce the quantity of fluid administered.

2 serrefine clamps were placed on each jugular vein, isolating segments ranging from 1 to 14 cm. and averaging 4 cm. in length. The presence of a thrombus forming a cast of the isolated segment was determined by removing one of the vein segments 10 minutes after isolation and examining its contents. Thrombi also invariably formed behind the distal clamp where stasis was partial.

Following confirmation of thrombus formation in one jugular vein, the length and diameter of the contralateral segment was measured and the proximal clamp on this vein removed, leaving the distal clamp *in situ*. The thrombus formed in the measured segment was eased proximally by gently raising the distal end of the vein; prompt movement of the thrombus toward the heart was readily visible through the vein wall. In 20 of these animals, tidal volume, minute volume, and rate of respiration were recorded on a kymograph attached to a Tissot spirometer before, during, and for 10 minutes after release of the thrombus. Simultaneous electrocardiographic tracings were obtained on a multichannel, direct writing recorder.

In a second group of 28 animals, a large volume of freshly formed thrombus was released into the venous circulation. Single clamps were placed on both isolated jugular veins, and in some instances on both femoral veins as well, within 60 seconds after eluate infusion. Extensive thrombosis occurred in the venous system behind these clamps. Ten minutes later the clamps were removed, and as much thrombus as possible was moved toward the heart by gentle massage of the neck and legs distal to the sites of clamping. In many of these animals serum eluate infusion followed by clamping of a vein and subsequent release of thrombi was repeated at 10-minute intervals as many as 8 times.

Three groups of control animals were also studied. One such group of 27 animals was treated in every respect like the experimental series except that the serrefine clamps were replaced by silk ligatures and the thrombi formed thus confined to their veins of origin. A second control group consisting of 14 dogs was subjected to eluate injection without vein isolation, while a third group of 13 dogs was subjected to vein isolation without eluate infusion.

All experimental and control animals were sacrificed by the intravenous injection of sodium pentobarbital at intervals from minutes to weeks after completion of the appropriate procedure. Each animal received 1 mg. of heparin per kg. of body weight 5 minutes before death to prevent postmortem clotting. In dogs sacrificed 12 or more hours after embolization, surgical asepsis was used. No antibiotic agents were administered.

At necropsy the heart and lungs of each animal were removed *en bloc* from the junctions of the venae cavae to the ascending aorta without loss of blood. The venous pathways from the areas of jugular vein isolation to the heart were dissected *in situ*. Blood was drained by gravity from the heart through a 40 μ sieve to retain small, loose thrombi. The right and left cardiac chambers were examined for macroscopically visible thrombi and the lungs for evidence of infarction. The pulmonary arteries were minutely dissected, and the size, location and gross appearance of all thrombi found were recorded. Each lobe was then sectioned transversely from apex to base at 5 mm. intervals and the cut surfaces examined grossly for evidence of pulmonary artery thrombosis. Recovered thrombi were fixed in 10 per cent formalin in isotonic saline for histologic examination. When no macroscopic thrombi were found, the distal portions of one or more lobes were similarly fixed and examined histologically for minute emboli in small peripheral pulmonary arteries. In several instances, large paraffin sections of entire lobar segments were also examined.

Results

Control Studies

Seventeen of the 27 dogs in which both jugular veins were isolated after serum eluate infusion, but in which the isolating clamps were

not removed, were sacrificed 15 to 120 minutes after eluate injection. Thrombi forming complete casts of the isolated segments were present in both jugular veins of all these animals. Examination of the venous pathways from these veins to the heart, the cardiac chambers and the pulmonary arteries revealed, in 2 animals, minute threadlike thrombi measuring 3 by 1.5 mm. or less within a small pulmonary artery branch. In the 10 remaining dogs, permitted to survive for 1 to 12 days following eluate injection, similar small pulmonary artery thrombi ranging from 1 to 25 mm. in length, and 0.5 to 2 mm. in diameter were found in 5 animals.

Among the 14 nonanesthetized dogs in which no surgical manipulation was performed but which were allowed to survive up to 3 days following an injection of serum eluate, filiform thrombi similar to those seen in the previous series were observed in 8.

In contrast with these findings, no trace of thrombus was found in the hearts or lungs of 12 of 13 animals that did not receive serum eluate but were subjected to anesthesia, venoclysis, and the systemic infusion of 30 ml. of isotonic saline followed by vein clamping. None of the 13 dogs showed evidence of thrombosis in any of the 13 isolated vein segments. In only one dog, sacrificed after 24 hours, was a single thrombus measuring 5 by 2 mm. found in a pulmonary artery. However, in 6 of the 13 animals in this group, including that in which the pulmonary thrombus was found, small thrombi were observed at necropsy in the foreleg veins that had served as the sites of venoclysis.

Embolization Studies With Single Measured Thrombi

Size of the Embolus Released. A series of 25 vein segments were measured with calipers; the contained thrombi were then removed and similarly measured. The average thrombus was 4 mm. shorter and 2 mm. narrower than its corresponding vein, and in no instance was the discrepancy in diameter greater than 3 mm. Of particular importance was the fact that no thrombus in this group was less than 4 mm. in diameter. Furthermore, such thrombi when measured *in vitro* at repeated intervals revealed no reduction in length or diameter for at least 4 hours after removal from the vein.

Results Obtained Within 4 Hours of Embolization. Twenty of the 40 dogs in which a single embolus was released were sacrificed within 4 hours of embolization (Table I). A red, nonadherent thrombus consistent with all, or part, of that originally released was found in the pulmonary arteries or in the right heart in every instance. In 10 of these animals, a single embolus consistent with that released was recovered intact. In the remaining 10, the embolus had fractured, as evidenced by

the uncovering of 2 to 4 pieces in 7 of these dogs, or by the finding, in 3, of a segment that could only have been a portion of the original embolus. Of those 7 animals in which more than one embolus was found, fragments were uncovered in opposite lungs in 3, in different lobes on the same side in 2, and in the right ventricle and a pulmonary artery in the remaining 2.

TABLE I

RECOVERY OF PULMONARY EMBOLI FOLLOWING RELEASE OF A SINGLE THROMBUS

Time of sacrifice after embolization	No. of dogs	No. of dogs in which emboli were recovered			No. with infarcts
		Total	In right heart	In pulmonary arteries	
3 min. to 4 hr.	20	20	8*	16	0
1 day to 28 days	20	10	1	10	0
1 to 4 days	6	6	1	6	
5 to 13 days	9	4	0	4	
15 to 28 days	5	0	0	0	
Total	40	30	9	26	0

* Four dogs revealed embolic fragments in a pulmonary artery as well.

All or part of the embolus was found in the right ventricle in 8 of the 20 animals. In 2 instances it was wedged securely between the ventricular wall and the chordae tendineae of the tricuspid valve (Fig. 1), and the chordae had left a distinct impression on the surface of the embolus. In one additional dog, an embolus was found in the pulmonary artery behind a cusp of the pulmonic valve.

In 6 of the 20 dogs, additional threadlike thrombi, similar to those observed in the control animals, and measuring less than 2 mm. in diameter, were also found in the pulmonary arteries or ventricular chambers. These thrombi, however, were readily distinguishable by their size and appearance from those released from the jugular vein.

No animal in this group showed any change in tidal volume, minute volume, rate of respiration, cardiac rate or rhythm, or electrocardiographic configuration during, or for 10 minutes after, release of the embolus.

Results Obtained 24 Hours or Longer After Embolization. Twenty additional dogs in which a single embolus was released were sacrificed 1 to 28 days after embolization (Table I). Although one or more small thrombi were found in each of the 6 animals sacrificed between 1 and 4 days, many of these measured less than 5 mm. in length and 1 mm. in

diameter, and could not be distinguished from similar minute thrombi noted in the control animals. In 4 of these 6 animals, however, additional larger thrombi measuring 2 to 3 mm. in diameter were also found in the pulmonary arteries, and, in one instance, in the right ventricle as well. These latter thrombi were believed to represent all or part of the emboli originally released and to have undergone a variable degree of reduction in size in the intervening period. All of these thrombi were red and nonadherent.

Of the 9 dogs sacrificed from 5 to 13 days after embolization, residual emboli were found in only 4. Of these 4 animals, adherent emboli up to 6 mm. in length and 1 mm. in width were present in 3, and the remaining nonadherent thrombus appeared to have been dislodged at the time of dissection. Microscopic examination of 2 of the larger of these emboli showed early organization at the periphery, with fibrin essentially free of red cells making up the bulk of the remainder (Fig. 2). In the 5 dogs sacrificed after 15 days or longer, no residual evidence of embolization could be found, nor was there any trace of the threadlike thrombi observed earlier. In those instances in which the lung tissue peripheral to the site at which emboli were found was examined histologically, no abnormalities were noted, nor were evidences of infarction, either gross or microscopic, observed.

With the exception of one thrombus found in the right ventricle 24 hours after release, no intracardiac emboli were noted in any animal of this series sacrificed after the first day.

Embolization Studies With Multiple Thrombi

Results Obtained Within 4 Hours of Embolization. Ten dogs were sacrificed within 4 hours of embolization (Table II). No emboli were

TABLE II

RECOVERY OF PULMONARY EMBOLI FOLLOWING RELEASE OF MULTIPLE THROMBI

Time of sacrifice after embolization	No. of dogs	No. of dogs in which emboli were recovered			No. with infarcts
		Total	In right heart	In pulmonary arteries	
3 min. to 4 hr.	10	10	5	10	0
1 day to 6 mo.	18	16	3	16	2
1 day	4	4	2	4	0
4 to 7 days	4	4	1	4	1
13 to 16 days	4	4	0	4	0
43 days to 6 mo.	6	4*	0	4*	1
Total	28	26	8	26	2

* One embolus found on microscopic examination only.

found in the venous pathways between the sites of vein isolation and the heart. The pulmonary arteries of all animals contained large amounts of dark red, nonadherent emboli, usually filling all the major pulmonary arteries (Fig. 3). Measurements of the recovered mass of thrombus were unreliable because of twisting, cohesion and friability, but estimates in several animals ranged from 40 to 100 cm. in total length. Nor was there any way of determining, during the course of an experiment, how much of a peripheral vein was actually emptied of thrombus at each release and massage, and, consequently, how much fresh thrombus was likely to form at the next clamping. The variability in the amount of emboli released was evident from the fact that, in several dogs, release of the contents of 2 femoral veins produced as great an apparent volume of pulmonary emboli as did 4 or more releases from 4 peripheral veins. The possibility also existed that pulmonary artery narrowing caused by previously released emboli may have produced sufficient stasis to induce local thrombosis upon injection of fresh serum eluate.

Emboli were also found in the chamber of the right ventricle in 5 of the 10 dogs in this group. In each instance the intracardiac emboli were enmeshed, totally or in part, in the chordae tendineae of the tricuspid valve.

Several animals in this group showed an increase in minute volume and rate of respiration. There were no significant changes in tidal volume, and alterations in cardiac rate and rhythm were minor. These and additional measurements of cardiopulmonary function will be reported in detail elsewhere.

Results Obtained 24 Hours or Longer After Embolization. Observations in the 18 dogs sacrificed 1 day to 6 months after embolization with multiple thrombi are summarized in Table II. No emboli were recovered in the venous pathways between the sites of vein isolation and the heart. Of the 12 animals sacrificed 1 to 16 days after embolization, emboli were found in the pulmonary arteries in every instance in contradistinction to the much smaller incidence of persistence in the animals subjected to a single embolus. Although there was some suggestion that the amount of thrombus in the pulmonary arteries was decreasing in volume during the first week, this could not be evaluated accurately in view of the unreliability of the measurements. After this first week, however, the decrease in the amount of residual emboli was striking, and in the 4 animals sacrificed between 13 and 16 days, scattered organizing thrombi not exceeding 5 cm. in total length were all that remained.

Traces of organized emboli were still to be found in 4 of the 6 animals sacrificed between 43 days and 6 months after embolization. In 3 of these—in one instance after 5 months—the original volume of thrombus released had been reduced to a few adherent nubbins which proved on

microscopic examination to be old, organizing emboli. In 2 specimens, the pattern of organization had produced strands resembling the "bridging" frequently observed in human pulmonary arteries at necropsy (Fig. 4), and changes suggesting an earlier phase of this phenomenon were noted in other organizing emboli (Fig. 2). In one additional animal, a small organized embolus, overlooked grossly, was found on microscopic examination. In the remaining 2 dogs, examined after 67 days and 6 months respectively, all traces of the original mass of thrombus had disappeared.

Pulmonary infarcts, small in proportion to the total amount of thrombus reaching the lungs, were noted in only 2 animals in this group. In one of these, sacrificed 7 days after embolization, 3 infarcts were found. These measured up to 2 by 1 cm., had a firm red appearance on gross examination, and presented the classical features of recent hemorrhagic infarction on microscopic examination. In the other animal, sacrificed 67 days after embolization, 2 small firm areas proved, on microscopic examination, to be old, organized infarcts even though no traces of the original emboli could be found.

One-half the animals sacrificed within the first 24 hours showed variable amounts of thrombi in the right ventricle as well as in the pulmonary arteries (Table II). In two of these the thrombi were entwined about the chordae tendineae of the tricuspid valve. A similar thrombus which had become adherent and showed microscopic evidence of early organization was also found in one animal sacrificed on the seventh day following massive embolization.

No spontaneous deaths occurred in any of the 28 animals subjected to massive embolization.

Discussion

The modifications of the technique of serum-induced thrombosis described in this communication provide a unique opportunity for study of the mechanism whereby autologous pulmonary emboli are handled by the animal. It is evident from the control data, as well as from previous studies,[2] that the method is highly reproducible and that interfering factors are minimal. Postmortem clots which might be confused with the experimental emboli are completely preventable by heparin, and the occasional filiform thrombi found in the hearts and pulmonary arteries of both control and experimental animals differ radically in size and appearance from those intentionally released. The relative roles of lysis and organization, as well as mechanical factors, in the behavior and eventual disposition of the experimental emboli can therefore be determined with a high degree of reliability.

When single measured emboli were released, thrombi consistent with

those sent off were invariably recovered in dogs examined within 4 hours after embolization. In view of the observed discrepancy between the vein measurement and the actual size of the thrombus released, no definite reduction in volume could be demonstrated within this period. After 24 hours, however, reduction in size became clearly evident with increasing time. Five days after embolization only a few residual emboli could be found, and after 2 weeks no traces were uncovered. Allowing for the possibility that a few of the residual emboli may have been lost or unrecognized in the course of dissection, it is apparent that a significant reduction in volume occurred, presumably by lysis.

Further evidence in support of the remarkable efficiency of thrombolysis was provided by those animals subjected to multiple emboli. From a volume of thrombus filling virtually every major radicle of the pulmonary arterial tree, a dramatic reduction to less than 5 cm. in total length was noted within 2 weeks. After 6 weeks, small organized nubbins were all that remained, and in some instances there was nothing left to indicate that a massive embolization to the lung had occurred.

No evidence of adherence between the embolus and the wall of the pulmonary artery was observed prior to the fifth day. All emboli persisting beyond this time, however, showed varying amounts of adherence and organization. When the volume of embolic material was small, as in the animals subjected to single emboli, about 30 per cent of the dogs sacrificed after the fifth day showed evidences of residual emboli. Although no trace of an embolus was found in this group after 2 weeks, the microscopic appearance of some of the organizing emboli observed earlier (Fig. 2) suggested that an occasional persistent fragment might have been observed beyond this time if the series had been larger, or that a nubbin partially incorporated into the wall of the vessel might have been overlooked. On the other hand, significant though markedly reduced amounts of thrombi were noted in every animal subjected to massive embolization and sacrificed within 16 days, and traces of organized embolic material were still present in one such animal after 5 months. It is apparent that persistence of emboli bears a direct, though obviously nonlinear, relation to the volume of thrombus reaching the lung. Furthermore, the effectiveness of the lytic mechanism, particularly in the first few days following embolization, appears to play a critical role in determining the amount of thrombotic material persisting long enough to undergo organization. Such local factors as the extent of contact of the embolus with the living tissue of the vessel wall undoubtedly also contribute to the rate and extent of the organization process.

The influence of certain mechanical factors on the behavior and fate

of pulmonary emboli is also evident from these experiments. In the group of animals subjected to a single embolus, fracture of the embolus occurred in one-half the dogs examined within the first 4 hours following release. The distribution of these fragments in different lungs or lobes, or in both heart and lung suggests that fracture occurred, in almost all instances, prior to entry of the embolus into the smaller pulmonary arterial branches and probably in the right heart itself. Although the possibility of fragmentation at the point of release was not entirely excluded, some additional observations make this unlikely. In several experiments, for example, thrombi released to narrowed portions of the veins proximal to the isolated segments were invariably intact in contrast with those permitted to reach the heart.

An additional mechanical factor was suggested by the presence of emboli in the chambers of the right heart. This delay in passage to the lung, though usually brief, was occasionally as long as 24 hours in the group subjected to single emboli. In those dogs subjected to massive embolization, emboli were found in the right heart after 7 days, and in one such animal not in this experimental series, there was evidence of an organized embolus in the right heart after 36 days.[3]

The infrequency and small size of the pulmonary infarcts found in these animals, and their occurrence only in dogs subjected to multiple emboli, is consistent with the general experience that infarcts are not readily produced in the presence of a normal bronchopulmonary circulation even when obstruction is extensive. Still more striking was the absence of spontaneous deaths, or even severe respiratory distress, either immediate or delayed, in any of the animals of this series. It is possible that transient right to left shunts, recently demonstrated in dogs subjected to massive embolization with serum-induced thrombi[4] may serve as a protective mechanism in ameliorating the hemodynamic load on the right heart. Survival of these animals may also be due in some measure to delay of emboli in the right heart, together with the remarkable efficiency of the lytic mechanism.

The source of the filiform thrombi found in the heart and pulmonary arteries of both control and experimental animals was frequently uncertain. Some of these thrombi occurring in the experimental group may have represented a portion of the embolus originally released from the jugular vein. Minute thrombi formed in the small ligated venous tributaries were occasionally included in the isolated venous segment and could be found attached to the control thrombus or to the recovered embolus itself. These small twigs could conceivably have been broken from the main body of an embolus in its passage to the lungs.

Such a mechanism, while possible in the experimental animals, cannot

explain the occurrence of these thrombi in the control groups. A source of such thrombo-emboli, common to both experimental and control groups, was the venoclysis site at which thrombi could be demonstrated in some instances even in the absence of eluate infusion. Of greater significance, however, seems to be the high degree of correlation between the occurrence of filiform thrombi and the infusion of serum eluate regardless of what else may have been done. Following such infusion, it is possible that thrombi of this type may form in areas of retarded blood flow in peripheral veins or pulmonary arteries. Supporting this concept is the fact that the incomplete thrombi which invariably form behind the second clamps occasionally resemble the filiform thrombi observed in the heart and lungs. Similar thrombi have also been produced in earlier experiments by simple external digital pressure on a peripheral vein following eluate infusion.[1]

Summary

The fate of serum-induced, autologous thrombi, released from peripheral veins as single or multiple emboli, was determined in a series of dogs by sacrificing the animals at intervals from minutes to months after embolization.

Marked reduction in the volume of the emboli was noted within the first 2 weeks, and no residua were observed after this time in dogs subjected to single emboli. Following massive embolization, traces of emboli persisted for longer periods; the effectiveness of thrombolysis, however, was such that a volume of thrombus filling virtually the entire pulmonary arterial tree was reduced after 6 weeks to a few small organized nubbins, or had entirely disappeared.

Adherence and organization of emboli were not noted prior to the fifth day, but were observed in every embolus encountered beyond this time.

Delay in passage of emboli through the right heart occurred in some instances, and such emboli occasionally persisted as endocardial thrombi. Fracture of emboli prior to entry into the pulmonary arteries was also observed in several animals.

No spontaneous deaths occurred. Rare pulmonary infarcts were observed only in those animals subjected to massive embolization, and, when present, were small in relation to the amount of emboli reaching the lung.

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Mrs. Carol Ho, Mrs. Helen Silver, and Mrs. Joan Moran provided valuable technical assistance.

[*Illustrations follow*]

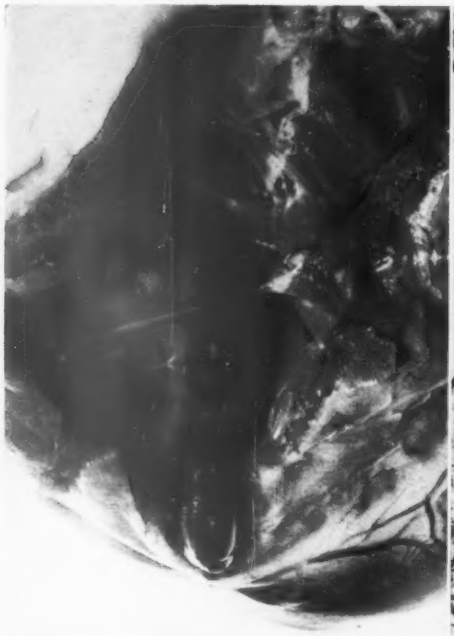
Legends for Figures

Fig. 1. An embolus recovered from the right ventricle 12 minutes after release. Note the well defined shape of the thrombus and compression by one of the chordae tendineae of the tricuspid valve.

Fig. 2. Early organization of a single embolus recovered from a pulmonary artery 15 days after release. Note organization into strands, suggesting an early "bridging" effect. Hematoxylin and eosin stain. × 58.

Fig. 3. Massive embolism to the lung. Dog sacrificed immediately following the release of multiple thrombi. Note that all pulmonary radicles are filled with thrombi.

Fig. 4. Strands of organized embolus resembling "bridging" in a pulmonary artery 95 days after massive embolization. Hematoxylin and eosin stain. × 50.



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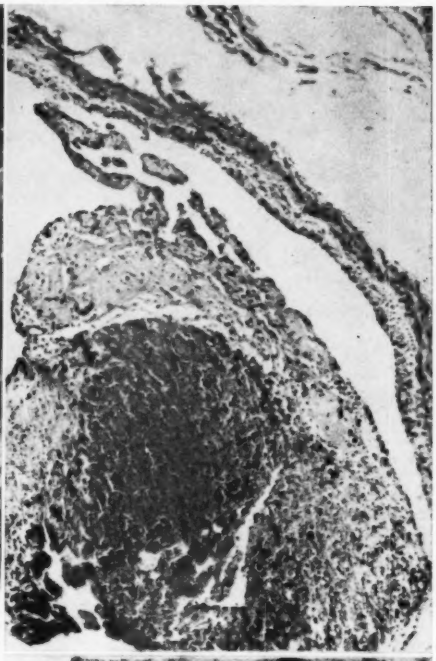

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THE EFFECT OF RUBIDIUM ON THE ADRENAL CORTEX OF NORMAL AND POTASSIUM-DEFICIENT RATS

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The group I alkali metal ions—potassium, rubidium and cesium—have similar physicochemical properties, and experimental evidence indicates that the first two act in an almost identical manner in various biologic processes. For example, rubidium may replace potassium as an essential element for the growth of bacteria,[1] produce effects similar to those of potassium on the resting potential of muscle,[2] and prevent myocardial necrosis in animals with potassium deficiency.[3,4] The present work offers evidence that rubidium may substitute for potassium in the maintenance of structure and presumably of function of the cells constituting the zona glomerulosa of the adrenal cortex.

MATERIAL AND METHODS

Male Wistar rats, weighing from 150 to 300 gm., were used in all of the experiments. One to 5 animals were housed in a single cage that was lined with a raised wire mesh to prevent ingestion of fecal matter. The animals were fed a basic diet deficient in potassium * and water *ad libitum*. In certain groups, potassium or rubidium chloride was added to the drinking water in concentrations of either 20 or 50 mEq/l.

Deoxycorticosterone acetate (DOCA) was suspended in a medium composed of 0.5 per cent carboxymethyl cellulose and 0.4 per cent Tween 80 in 0.9 per cent sodium chloride (the DOCA and suspending vehicle were generously supplied by the Lederle Laboratories). Two tenths ml. of this suspension, containing 2 mg. of DOCA, was injected subcutaneously each day at the base of the tail. The administration of this agent served further to deplete the animals of potassium.

Plasma potassium determinations were performed on blood collected in heparinized syringes by cardiac puncture. Determinations were done with the Advanced Flame Photometer, Model 11B. Plasma potassium values on 40 normal rats ranged from 4 to 5.6 mEq/l., with an average of 4.7 mEq/l.

Unilateral adrenalectomy was performed on animals under light anesthesia with ether or sodium pentobarbital. The adrenal gland was removed through a vertical incision made below the subcostal margin on the dorsal aspect of the left side. All wounds healed without evidence of infection.

Necropsy examinations were carried out after the animals died or had been sacrificed. Tissues were fixed in 10 per cent formalin buffered to a pH of 7. Sections of the heart, adrenal gland, and kidney were stained with hematoxylin and eosin, and,

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* The potassium-deficient diet, manufactured by Nutritional Biochemicals Corp., was composed of cornstarch, 64.2 per cent; casein, 30 per cent; butter fat, 3.5 per cent; calcium carbonate, 1.3 per cent; sodium chloride, 1 per cent; plus complete vitamin supplements.

in addition, the last of these tissues was treated with the periodic acid-Schiff stain. Frozen sections of the adrenal gland were cut at 15 μ and stained with oil red O for neutral fats. Unstained companion sections from certain of the groups were examined under the polarizing and ultraviolet microscopes. For this technique, control sections were washed overnight in acetone to remove cortical lipids. Glycerol-gelatin was employed throughout as a mounting medium.

Effect of Rubidium on Adrenal Cortical Lipid Distribution in Normal and Potassium-Deficient Rats

The general plan of the experiment was as follows: As revealed by oil red O stains, the adrenal lipids of normal and potassium-depleted rats were compared with those of similarly treated animals that were given rubidium supplements. Potassium depletion of selected animals in the various groups was evaluated by means of plasma potassium determinations and observation of structural changes in the heart and kidney. These chemical and anatomic studies indicated that the rats fed the potassium-deficient diet were actually depleted of potassium.

Table I

DISTRIBUTION OF ADRENAL CORTICAL LIPIDS AND INCIDENCE OF MYOCARDIAL AND RENAL LESIONS IN RATS FED A POTASSIUM-DEFICIENT DIET SUPPLEMENTED WITH RUBIDIUM

GROUP	NO. OF RATS	TREATMENT	ADRENAL LIPIDS*	MYOCARDIAL NECROSIS	RENAL LESIONS
1 CONTROL	9	POTASSIUM		NIL	NIL
2	5			MARKED	MARKED
3	10	DOCA		MARKED	MARKED
4	9	RUBIDIUM		NIL	NIL
5	8	RUBIDIUM DOCA		MINIMAL	NIL
6	5	RUBIDIUM POTASSIUM		MODERATE	NIL

*AS SHOWN BY OIL RED O STAINING
■ – NORMAL LIPID CONTENT
■ – MODERATE DEPLETION OF LIPID
□ – MARKED DEPLETION OF LIPID
1–ZONA GLOMERULOSA
2–CHROMOPHOBIC BAND
3–ZONA FASCICULATA AND RETICULARIS
4–MEDULLA

As is shown in Table I, these experiments make it clear that the administration of rubidium prevented the depletion of lipid in the zona glomerulosa otherwise induced by potassium deficiency. This action of

rubidium on the zona glomerulosa was considered to be intimately related to the substitution of rubidium for potassium. In addition, rubidium produced another effect on the adrenal; namely, a marked loss of lipid in the zona fasciculata and zona reticularis. The experimental data will now be considered in detail.

Plasma Potassium Values

The plasma potassium values were uniformly diminished (2.8, 2.9, 2.9, and 3.0 mEq/l.) in 4 animals fed the deficient diet and given daily injections of DOCA for 29 days. Except for one animal which was sacrificed after having been on the diet for a brief interval of time, the plasma potassium values were similarly diminished in rats fed the potassium-deficient diet alone. In this group, the plasma potassium values were 2.7, 2.9, 3.1, and 4.3 mEq/l. for 4 animals that had been on the diet 35, 35, 28, and 11 days, respectively. After the animals were bled they were sacrificed. All animals with diminished plasma potassium values showed lesions characteristic of potassium deficiency in the adrenal, heart, and kidney, while the animal with a normal plasma potassium did not exhibit these alterations. The details of the anatomic lesions in the heart and kidney will be described below. The results indicate that rats placed on a potassium-deficient regimen were depleted of potassium.

Normal Cortical Lipid Distribution

As is shown in Figure 1 and schematically in Table I, the cells comprising the zona glomerulosa of the 9 control animals fed the potassium-deficient diet and receiving potassium supplements containing 20 to 50 mEq/l., stained intensely and uniformly with oil red O. The cells of the zona fasciculata immediately internal to the zona glomerulosa failed to take up the oil red O dye, thus forming a chromophobic band that stood in sharp contrast to the remainder of the zona fasciculata which stained intensely. The staining characteristics of the zona reticularis were similar to those of the zona fasciculata except for the portion immediately adjacent to the medulla, which stained less intensely.

Cortical Lipid Distribution in Potassium Deficient Animals

Lipid was virtually absent in the zona glomerulosa of each of the 10 animals in group 3 treated with a diet deficient in potassium and with daily injections of DOCA for 11 to 29 days (Table I and Fig. 2). It is of interest that 6 rats sacrificed as early as the eleventh to 13th days had marked depletion of lipid in the zona glomerulosa. In contrast,

the zona fasciculata and zona reticularis of all the animals contained normal amounts of lipid, as judged by oil red O preparations. Depletion of lipid from the zona glomerulosa was similar but less extreme in 4 of the 5 animals in group 2, the rats that were fed the potassium-deficient diet alone for periods ranging from 11 to 35 days. As indicated previously, one animal placed on the potassium-deficient diet for only 11 days did not develop hypokalemia or alterations in the adrenal cortex.

The Effect of Rubidium on the Adrenal Cortex of Potassium Deficient Rats

The 17 rats in groups 4 and 5, which were depleted of potassium and simultaneously treated with rubidium, did not show the loss of lipid in the zona glomerulosa found in potassium-deficient rats that did not receive this supplement. In addition to preventing the changes in the zona glomerulosa, rubidium caused marked reduction of lipid in the zona fasciculata and zona reticularis.

The 8 rats in group 5 were depleted of potassium by dietary means and by administration of DOCA for 11 to 17 days and in addition received 50 mEq/l. of rubidium in the drinking water and 1 mEq intraperitoneally. There was an intense affinity of the zona glomerulosa for the oil red O dye (Table I; Fig. 3). The zona fasciculata and zona reticularis were wider than in normal controls, and presumably this enlargement brought about the increased size of the adrenal noted grossly. Concomitant with this enlargement, there was a marked depletion of lipid in these zones in 7 of the 8 animals.

The findings in the adrenal were similar in the 9 rats of group 4 that were treated with the potassium-deficient diet alone supplemented with either 20 or 50 mEq/l. of rubidium in the drinking water for 9 to 28 days. The zona glomerulosa stained intensely with the oil red O dye in all animals. The inner zones were enlarged and devoid of lipid in 6 of the animals, while 2 adrenals showed a normal distribution of lipid and 1 contained lipid in all zones but failed to show the chromophobic band normally separating these zones.

The Effect of Rubidium on the Adrenal Cortex of Normal Rats

An additional control group of 5 rats (Table I, group 6) were fed the basic potassium-deficient diet but were given drinking water containing 20 mEq/l. of both potassium and rubidium chloride. When the animals were sacrificed after 20 days, the adrenals showed changes similar to those in the previous groups given rubidium. In each rat the zona fasciculata and zona reticularis were enlarged but depleted of lipid, while the zona glomerulosa stained intensely with oil red O.

Effects of Rubidium on Adrenal Cortical Lipids, Birefringence, and Autofluorescence in Normal and Potassium-Deficient, Unilaterally Adrenalectomized Rats

The effect of rubidium on the zona glomerulosa of the adrenal of individual rats was studied by removing one of the adrenals of either a potassium-deficient or a normal animal, and then feeding rubidium and the potassium-deficient diet for varying intervals of time postoperatively. The cortical lipids, birefringence, and autofluorescence of the gland removed before rubidium administration were then compared with those in the adrenal remaining in the animal after rubidium was added to the diet. Control observations were made to see whether or not the surgical procedure itself induced similar changes in the remaining adrenal of animals receiving adequate supplements of potassium.

Effect of Unilateral Adrenalectomy on the Remaining Adrenal of Normal Rats

The results indicated that the removal of one adrenal failed to alter the distribution of lipid, autofluorescence, and birefringence in the remaining adrenal of normal animals under the conditions of the present experiment. The histologic observations on the control group are summarized in Table II.

Five rats were placed on the basic potassium-deficient diet supple-

Table II

DISTRIBUTION OF ADRENAL CORTICAL BIREFRINGENCE BEFORE AND AFTER TREATMENT WITH RUBIDIUM IN RATS FED A POTASSIUM-DEFICIENT DIET

GROUP	NO. RATS	TREATMENT	UNILATERAL ADRENALECTOMY		POST-OP	AUTOPSY
			day	adrenal lipids *	treatment - days	adrenal lipids *
1 CONTROL	5	POTASSIUM	26		POTASSIUM 16	
2	5	POTASSIUM	54		RUBIDIUM 14-27	
3	6		40		RUBIDIUM 7-21	
4	10	DOCA	13-15		RUBIDIUM DOCA 8-13	

*AS SHOWN BY BIREFRINGENCE
NORMAL
MARKEDLY REDUCED
1-ZONA GLOMERULOSA
2-CHROMOPHOBIC BAND
3-ZONA FASCICULATA AND RETICULARIS
4-MEDULLA

mented with 20 mEq/l. of potassium in the drinking water. This regimen was continued throughout the entire experiment. On the 26th day unilateral adrenalectomy was performed, and 16 days later all animals were sacrificed and the remaining adrenal removed. The pattern of lipid distribution was similar in glands removed operatively and at necropsy.

All 10 adrenals in the 5 control rats showed a normal pattern of lipid distribution when stained with oil red O. Likewise, the pattern of birefringence was similar in all 10 adrenals. The zona glomerulosa appeared as a thin band beneath the capsule and contained a uniform distribution of birefringent crystals which appeared white against a black background (Fig. 4). The majority of crystals were fine or medium, but there were occasional coarse ones. Between the zona glomerulosa and the zona fasciculata was a photophobic band that corresponded to the chromophobic band seen in sections stained with oil red O. The zona fasciculata and the zona reticularis appeared as a single broad band delimited by the photophobic band externally and the medulla internally. The majority of the crystals dispersed throughout the zona fasciculata and zona reticularis were fine or medium. There appeared to be a greater number of fine crystals in the inner zones of the adrenals of animals previously subjected to unilateral adrenalectomy.

Unstained sections mounted in glycerol-gelatin were examined with the ultraviolet microscope (Zeiss-Winkler). A #1 (BG 12 Schott) exciter filter was used for observation of fluorescent light in the range of 5,000 to 8,000 Å. The autofluorescence emitted from the gland appeared as a green-yellow light. There were no consistent differences between glands removed at the time of unilateral adrenalectomy and at necropsy. The zona glomerulosa, zona fasciculata and the zona reticularis contained a large quantity of autofluorescent material.

The Effects of a Potassium Deficient Diet Supplemented with Rubidium in Normal Animals

The results of this experiment showed that rubidium supplements fed simultaneously with a potassium-deficient diet prevented the histologic alterations in the zona glomerulosa previously described in potassium-depleted animals. The zona fasciculata and zona reticularis of rubidium-treated animals were enlarged and showed a marked loss of lipid (Table II, group 2).

Five rats were placed on the basic potassium-deficient diet supplemented with oral potassium (50 mEq/l.) for a period of 54 days prior to unilateral adrenalectomy. Postoperatively, oral rubidium (50 mEq/l.) was substituted for potassium and the basic diet was con-

tinued. The animals were sacrificed or died 14 to 27 days postoperatively, and the adrenals were compared.

Since the rats received an adequate intake of potassium prior to operation, the adrenal removed surgically served as a normal control for each animal. As anticipated, these adrenals had a normal distribution and amount of lipid, birefringence, and autofluorescence. A comparison of these controls with the zona glomerulosa in the same animals after rubidium was substituted for potassium, failed to demonstrate significant differences. In each animal, the zona glomerulosa had a marked affinity for the oil red O dye, contained many birefringent crystals, and was strongly positive for autofluorescent material.

In contrast, the zona reticularis and zona fasciculata at the time of necropsy displayed marked alterations (Table II). These zones were wider than normal and uniformly exhibited a marked reduction of oil red O-positive material. In 3 instances these zones were virtually devoid of oil red O-positive substance while in 2 animals the changes were somewhat less marked. There was also a reduction of birefringence paralleling the loss of lipid. The diminution in birefringent crystals was extreme in 4 instances and moderate in one. Studies with the ultraviolet microscope did not show a close correlation with the alterations described above. There was a loss of autofluorescence in only one animal; the adrenals in the remaining animals showed no observable differences from the surgically removed control glands.

The Effects of Feeding Rubidium to Potassium-Deficient Animals

The purpose of the experiment with this group was to learn whether the feeding of rubidium supplements would bring about a reversal of the alterations in the zona glomerulosa of an animal previously depleted of potassium by a deficient diet (Table II, group 3).

Six rats were fed the basic potassium-deficient diet for 40 days. The left adrenal was then removed surgically; thereafter, rubidium supplements (50 mEq/l.), were added to the drinking water and the basic potassium-deficient diet was continued. The animals died or were sacrificed 7 to 21 days postoperatively, and the adrenals were compared with those removed surgically.

The distribution of lipid in the operatively removed adrenal was characteristic of potassium deficiency. The zona glomerulosa was atrophic and depleted of birefringence in 6 sections, of fluorescence in 6, and of oil red O-positive material in 2. There was no alteration in the lipid content of the zona fasciculata and zona reticularis. After treatment with rubidium there was a reversal of the lipid pattern. The zona glomerulosa exhibited an increased amount of birefringence in 5 sec-

tions, fluorescence in 6, and oil red O-positive material in 2. In contrast, the zona fasciculata and the zona reticularis were enlarged and depleted of birefringence in 6 sections, of fluorescence in 2, and of oil red O-positive material in 6 (Table II; Figs. 5 and 6).

The Effect of Rubidium on the Adrenal Cortex of Rats Fed a Potassium-Deficient Diet Augmented with DOCA, Prior to Unilateral Adrenalectomy

The experimental results were similar to those in the previous group (Table II). Treatment prior to adrenalectomy with a potassium-deficient diet augmented with DOCA induced changes in the zona glomerulosa selectively. The addition of rubidium reversed the alterations in this zone but did induce changes in the zona fasciculata and zona reticularis.

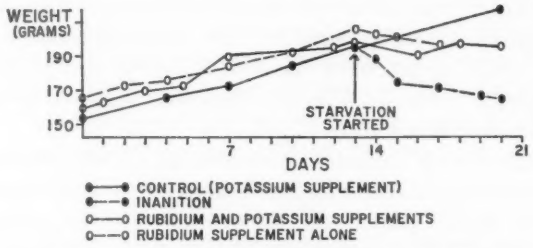
Ten rats were placed on a potassium-deficient diet augmented with daily subcutaneous injections of 2 mg. of DOCA. Unilateral adrenalectomy was performed on days 13 to 15. Fifty mEq/l. of rubidium chloride was then added to the drinking water and 1 mEq per day was given intraperitoneally. The potassium-deficient diet and daily injection of DOCA were continued. This regimen was continued for 8 to 13 days; the animals were then sacrificed.

The alterations in the adrenal removed operatively were similar to those produced by a potassium-deficient diet alone but were more extreme. The zona glomerulosa exhibited marked lipid depletion in 10 instances and reduction of birefringence in 10 and of autofluorescence in 7. In contrast, the zona fasciculata and the zona reticularis stained intensely with oil red O and contained large amounts of autofluorescent material and many birefringent crystals of fine to medium size. The opposite adrenal which was removed after treatment with rubidium showed a reversal of the above pattern. The zona glomerulosa revealed repletion of total lipid (as determined by oil red O stains) in 6 adrenals, of birefringence in 8, and of autofluorescence in 5. In contrast to the zona glomerulosa, the inner zones were depleted of lipid in 8 instances, of birefringent substance in 7, and of autofluorescent material in one.

Thus, despite the fact that significant alterations had been induced in the zona glomerulosa at the time of adrenalectomy and the potassium-deficient diet and daily injections of DOCA were continued throughout the entire experiment, rubidium was able to prevent further alterations and actually promoted the repletion of material in the outer zone. In addition, it induced alterations in the zona fasciculata and zona reticularis.

Effect of Inanition on the Adrenal Cortex

Previous reports have shown that weight loss may be associated with alterations in the adrenal cortex.[5] Since some degree of weight loss was common in rats receiving rubidium, the following experiment was performed to see whether or not weight loss *per se* was responsible for the changes in the zona fasciculata and zona reticularis in animals fed rubidium (Text-fig. 1).



Text-figure 1. Effect of rubidium and of inanition on weight of rats.

A control group (10 male rats) was fed the potassium-deficient diet supplemented with 20 mEq/l. of potassium in the drinking water. After 13 days, 5 of these animals were continued on this regimen as a control group, whereas the remaining 5 rats were deprived of all food but allowed free access to distilled water. A third group of 5 rats received the potassium-deficient diet supplemented with both potassium (20 mEq/l.) and rubidium (20 mEq/l.) in the drinking water. A fourth group of 5 rats received the potassium-deficient diet supplemented with rubidium (50 mEq/l.) alone. All animals were sacrificed on the 20th day, except for 3 in group 4 receiving rubidium supplements alone. These died on the 14th, 18th, and 18th days, respectively.

The animals were weighed at frequent intervals. The average weight in each group is plotted in Text-figure 1. The rats in the control group gained an average of 3.2 gm. per day for 13 days, and the weight gain in the other groups receiving rubidium was essentially similar. Thereafter, marked differences were noted in the various groups. Control animals continued to gain an average of 3.2 gm. per day, while rats deprived of food lost an average of 3.8 gm. per day. After the 13th day, the animals receiving rubidium showed disturbances in weight gain when compared with the controls. Those fed rubidium and a diet containing adequate quantities of potassium (group 3) failed to gain weight, while the

potassium-deficient animals receiving rubidium supplements (group 4) lost weight at the average rate of 1.2 gm. per day.

Animals deprived of all food for 13 days exhibited only very minor alterations in the lipid distribution in the adrenal cortex as shown by oil red O staining. The zona glomerulosa appeared normal in all 5 animals, and the zona reticularis and zona fasciculata appeared normal in 4 of 5 instances; the exception showed only moderate depletion of lipid. A minor alteration observed in the adrenals of 2 animals subjected to starvation was a loss of the chromophobic band. The changes in the animals fed rubidium were similar to those previously described. They consisted of enlargement of the zona fasciculata and zona reticularis, with moderate to marked depletion of lipid in 8 of 10 animals (groups 3 and 4). The distribution of lipid in the adrenals of the control groups was normal.

These experiments indicated that after an initial period of normal growth, failure to gain or actual loss in weight occurred if rubidium was added to the diet of rats. Under the conditions of the experiment, the weight loss in rubidium-treated animals was much less than that found in rats deprived of food. In contradistinction, the changes in the zona fasciculata and zona reticularis in the animals deprived of food were negligible when compared to the animals receiving rubidium. The experiment indicated that weight loss *per se* in the rubidium-treated animals did not bring about the alterations in the zona fasciculata or zona reticularis.

Renal Tubular Lesions

The renal lesions in rats depleted of potassium by deficient diets alone or deficient diets and DOCA were similar to those described by Oliver and colleagues.[6] These consisted of necrosis and regeneration of the epithelium lining the collecting tubules in the outer portion of the medulla, and an accumulation of periodic acid-Schiff (PAS) positive granules in the cells lining the tubules of the renal papilla. These alterations in the outer medulla and in the papillas of the rats in the various experimental groups were arbitrarily graded (Table I). The results indicated that the addition of rubidium supplements to the diets of the potassium-deficient animals prevented the development of the renal lesions. These observations extend those of Follis[4] who showed that rubidium prevented tubular dilatation, accumulation of lipid droplets, necrosis, and regeneration in the renal convoluted tubules of potassium-deficient rats.

Myocardial Necrosis

Focal necrosis of the myocardium is a well recognized change in potassium-deficient animals.[7] The following observations confirm and

somewhat extend earlier work which indicated that rubidium may prevent such lesions.[4]

Myocardial necrosis was quite extensive in 14 of the 15 animals in groups 2 and 3 that were placed on the potassium-deficient diet alone or the deficient diet and DOCA (Table I). It seems significant that the single animal that did not have myocardial necrosis was also the only one that did not show lowered plasma potassium levels.

In striking contrast, the rats in group 4 that received rubidium supplements failed to develop myocardial necrosis. The results were less uniform in group 5 in which DOCA was used to augment the effects of the potassium-deficient diet, and where the myocardium of 4 of the 8 rats contained small foci of necrosis. Paradoxically, myocardial necrosis was observed in 4 of 5 rats in group 6 despite the fact that they were not depleted of potassium. Follis reported similar findings in 3 rats, and as in the present experiments the pathogenesis was obscure.

Discussion

These experiments show that the addition of rubidium to a potassium-deficient diet prevents or reverses the atrophy and the loss of lipid, birefringence, and autofluorescence of the zona glomerulosa of the adrenal cortex otherwise characteristic of potassium deficiency in rats. They suggest that rubidium is capable of replacing potassium in the regulation of the zona glomerulosa and re-emphasize the importance of potassium in the regulation of this zone under physiologic circumstances.

The factors concerned in the regulation of the zona glomerulosa and their relationship to one another are not fully understood. Deane, Shaw and Greep [8] demonstrated that an increase in the ratio of sodium to potassium resulted in atrophy of the zona glomerulosa; a decrease in the ratio of sodium to potassium resulted in hypertrophy of this zone. Subsequently, perfusion experiments using the isolated adrenal of the cow showed that decreasing the ratio of sodium to potassium resulted in an increased secretion of aldosterone.[9] Other workers have correlated hypertrophy of the zona glomerulosa with increased aldosterone production in the rat.[10,11] Other investigations indicate that potassium *per se* may be an effective stimulus for the secretion of aldosterone.[12] A tropic hormone,[13] elevated venous pressure,[14] and intra-arterial blood volume [15] are other factors which may be important in the physiologic regulation of the zona glomerulosa.

In the present experiments, it seems likely that the following occurred. Administration of a diet deficient in potassium increased the ratio of sodium to potassium and resulted in an atrophy and loss of lipid in the zona glomerulosa and a decreased production of aldosterone. Rubidium supplements restored the ratio to normal, since this ion served as a

substitute for potassium. As a result, the histologic alterations in the zona glomerulosa were either prevented or reversed. If potassium alone serves as an effective stimulus for the secretion of aldosterone, the changes in the zona glomerulosa may be explained simply on the basis of an increase or a decrease in the concentration of potassium or its surrogate, rubidium.

The precise manner in which rubidium substituted for potassium can only be surmised. For example, rubidium may have replaced potassium, ion for ion, in both the intra- and extra-cellular compartments. It seems more likely, however, that rubidium selectively displaced intracellular potassium, for Relman, Lambie, Burrows and Roy[16] have demonstrated that rubidium accumulates intracellularly in skeletal muscle. Presumably the intracellular accumulation of rubidium displaces potassium, for Kunin, Dearborn and Relman[17] have shown that the infusion of rubidium into normal female dogs results in an increase in the serum concentration of potassium.

So-called toxic manifestations were noted in the rats given rubidium, i.e., weight loss, convulsions, and eventually death. In this relation, it is of interest to note that the adrenals were grossly enlarged. Microscopic examination revealed this to be due to an increase in the size of the zona fasciculata and zona reticularis while these zones were at the same time depleted of lipid, birefringence, and autofluorescence. Similar changes have been reported as a nonspecific reaction to stress in a variety of circumstances such as extreme weight loss, thiamine deficiency, and exposure to heat or cold, etc.[5,18–20] Presumably, the changes in this experiment were of a similar nature.

Summary

Rats depleted of potassium develop atrophy and loss of lipid, birefringence, and autofluorescence in the zona glomerulosa of the adrenal cortex. Supplementation of the potassium-deficient regimen with rubidium prevented these alterations in the zona glomerulosa and reversed them in rats with established potassium deficiency. The significance of these observations in relation to the structure and function of the zona glomerulosa has been discussed. In addition, rubidium caused an enlargement and loss of lipid in the zona fasciculata and zona reticularis of normal and potassium-deficient rats. This was considered to be a nonspecific secondary reaction.

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LEGENDS FOR FIGURES

FIG. 1. Adrenal gland from a control rat fed a potassium-deficient diet and receiving potassium (50 mEq/l.) as a sole source of drinking water for 54 days. The outer zona glomerulosa and inner zona fasciculata-reticularis stain intensely with oil red O and are separated by a chromophobic band. Oil red O stain. × 80.

FIG. 2. Adrenal gland of a rat fed a potassium-deficient diet augmented with DOCA for 11 days. Note atrophy and depletion of lipid in the zona glomerulosa. The zona fasciculata and zona reticularis stain intensely for lipid. Oil red O stain. × 80.

FIG. 3. Adrenal gland of a rat fed a potassium-deficient diet augmented with DOCA and supplemented with rubidium for 12 days. Note that the zona glomerulosa contains large amounts of lipid and that the zona fasciculata and zona reticularis are enlarged and depleted of lipid. Oil red O stain. × 80.

FIG. 4. Adrenal gland from a control rat fed a potassium-deficient diet and receiving potassium (50 mEq/l.) as a sole source of drinking water for 54 days. Note that all zones contain many birefringent crystals. The zona fasciculata and zona reticularis are bounded externally by a photophobic band and internally by the medulla. Polarized light, unstained frozen section. × 80.

FIG. 5. Adrenal gland removed surgically from a rat fed a potassium-deficient diet for 40 days. Note that the zona glomerulosa is depleted of birefringent material while the zona fasciculata and zona reticularis contain many medium-sized birefringent crystals. Polarized light, unstained frozen section. × 80.

FIG. 6. Remaining adrenal of the rat shown in Figure 5 after treatment with rubidium for 7 days. The zona glomerulosa contains many fine birefringent crystals while the zona fasciculata and zona reticularis are enlarged and depleted of birefringent crystals. Polarized light, unstained frozen section. × 80.

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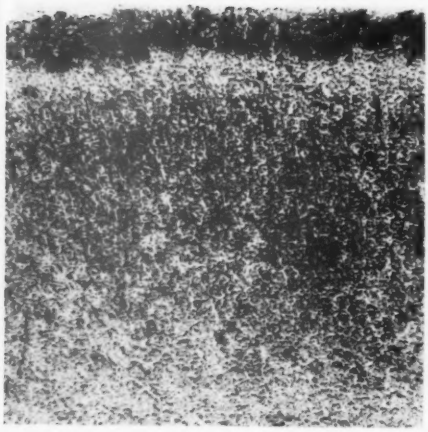

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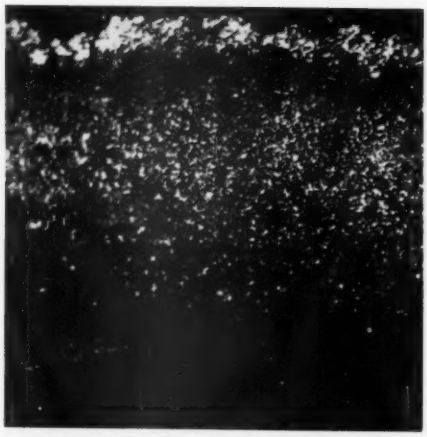

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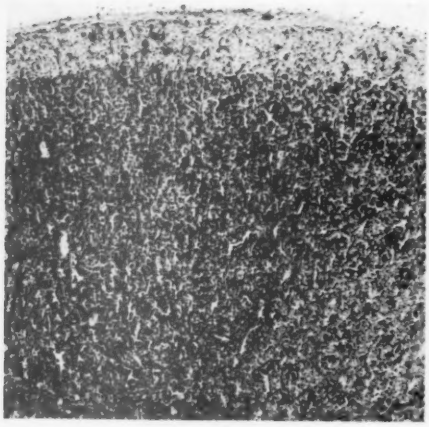

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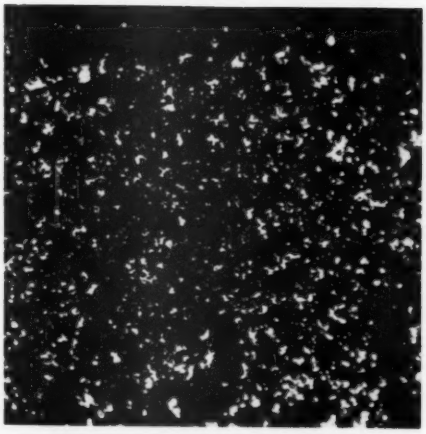

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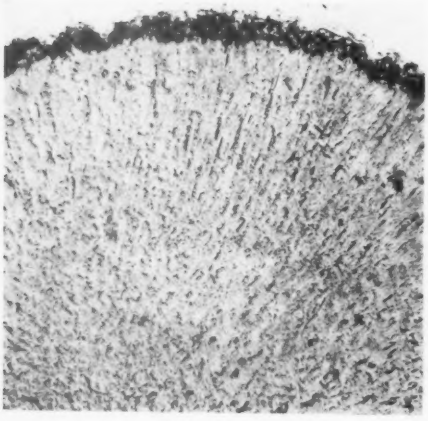

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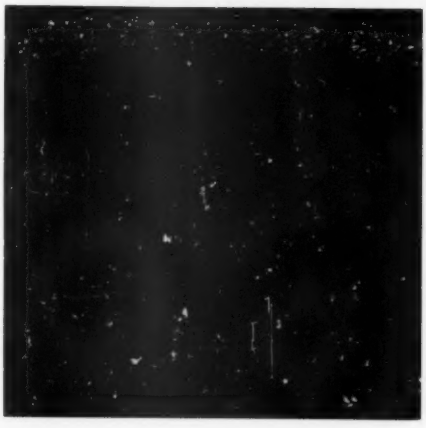

6

VIRUS-B INFECTION OF THE CENTRAL NERVOUS SYSTEM OF MONKEYS USED FOR THE POLIOMYELITIS VACCINE SAFETY TEST

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In 1934, Sabin and Wright[1] isolated a virus from the brain and spinal cord of a patient dying of acute transverse myelitis. The patient had been bitten by an apparently normal rhesus monkey and had become ill shortly thereafter. This virus was called "B-virus"* and has been studied quite extensively. Although, initially, there was no success in transmitting the disease to rhesus monkeys, Sabin[2] was later able to infect them with the virus. In 1935, Sabin and Hurst[3] described the pathologic features of experimental Virus-B infection in rhesus monkeys. They found that animals inoculated intracerebrally developed severe meningitis with lesions developing around the penetrating blood vessels. In one of 7 inoculated animals, there were glial and necrotic foci in the white and gray substance of the brain.

A recent report has described the lesions of natural Virus-B infection in monkeys.[5] The lesions in the central nervous system were minimal, consisting of localized involvement of the pons and medulla. There were glial and lymphocytic infiltrations around the nerve roots of the trigeminal and facial nerves and in the nucleus and tract of the descending branch of the trigeminal nerve and in the solitary tract. It is our belief that this histologic pattern may be altered and increased in severity by artificial means. We wish to report on Virus-B infection as we have seen it in the course of examining histologic preparations of central nervous system tissue of monkeys used in the safety test for poliomyelitis vaccine.[6]

MATERIAL AND METHODS

Monkey Safety Test for Poliomyelitis Vaccine

Rhesus (*Macaca mulatta*) or cynomolgus (*Macaca irus*) monkeys, weighing 4 to 8 pounds and in overt good health, were used. Pre-inoculation blood samples were obtained from the femoral vein. Animals were anesthetized with sodium pentobarbital (15 mg. per lb.) given intraperitoneally; 200 mg. of cortisone acetate were given in the left thigh and calf muscles, and 300,000 units of procaine penicillin were injected in the right deltoid muscle region.

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* Various names have been used to designate this virus; among them, B-virus, Virus-B, *Herpesvirus simiae*,[4] herpes B virus. In this paper the term Virus-B will be used.

Poliomyelitis vaccine was inoculated by the combined intraspinal, intracerebral and intramuscular routes as follows: 0.5 ml. into the lumbar enlargement of the spinal cord, 0.5 ml. into the thalamic region of each cerebral hemisphere and 1 ml. into the gastrocnemius muscle of the right leg. Animals were observed daily for 17 to 19 days for signs of poliomyelitis. At the end of this period, the monkeys were sacrificed by bleeding from the heart, and a 10 ml. blood sample was saved as the post-inoculation specimen.

Necropsy Observations

The spinal cords were removed by the dorsal approach. Blocks of tissue from the lumbar and cervical enlargements and the cerebral cortex at the site of injection were removed aseptically and frozen, in the event that virus isolation studies were indicated. The monkeys were then perfused through the arteries with 10 per cent formalin saline solution.

Initially, histologic examination was carried out on tissues from the lumbar and cervical spinal cord. If lesions were found, tissue from the medulla, pons, midbrain, cerebellum and cerebrum were also studied.

Sections were stained with gallocyanin[7] and with azure-eosin.[8]

Virologic Techniques

Virus isolation attempts were made on tissues from all animals with histologic lesions. Sections of lumbar and cervical cord and frontal cortex were pooled, and a 20 per cent suspension was prepared in Eagle's basal medium containing antibiotic agents.

Rhesus monkey kidney cell cultures and rabbit kidney cell cultures were trypsinized according to methods described by Youngner.[9] Growth medium consisted of Medium 199 with 2 per cent calf serum; maintenance medium was Eagle's basal medium. Cultures were washed 3 times with Earle's balanced salt solution before inoculation.

One tenth of a ml. of the tissue suspension was inoculated into both monkey and rabbit kidney cell cultures containing 1 ml. of maintenance medium. Cultures were incubated at 36° C. and observed daily for 14 days, with sub-cultures being made at the end of 7 days. Supernatant fluids from tubes manifesting cell degeneration were transferred to fresh cultures to confirm the presence of a transmissible agent.

Neutralization tests were performed in rabbit kidney cell cultures against approximately 100 $TCID_{50}$ with specific anti Virus-B serums.

Results

During the period 1957 to 1959, the spinal cords of 6,300 monkeys were examined, and 26 were found to have lesions of various types. In 5 of the 26, the lesions were quite similar. Attempts were made to isolate agents from the spinal cords in all 26 monkeys. This was successful only in the 5 cases which had similar histologic alterations. The agents recovered from these 5 monkeys were all identified as Virus-B by specific type of cytopathogenic effect, neutralization by Virus-B antiserums (4 cases) and, in one instance, by rabbit inoculation.

Histologic Observations

There was a diffuse encephalomyelitis in all 5 monkeys. In the lumbar cord, there was severe necrosis, most marked in the area of inoculation

trauma (Figs. 1 and 2). The architecture of the lumbar cord was destroyed at one or more levels in all the animals. There was a lymphocytic infiltration of the meninges; the nerve roots surrounding the lumbar levels were infiltrated by neutrophils and there was focal demyelinization (Fig. 3).

In the thoracic and cervical cord, damage was much less severe. There were lymphocytic infiltrations around penetrating blood vessels and small glial foci in the gray and white substance (Fig. 4). Neurons were not damaged in the cervical levels, whereas, in the lumbar cord, the necrotizing process had destroyed them.

Lesions were quite extensive in the medulla and pons. At these levels the lesions were midline in all cases and there was marked edema of the tissue with necrosis and a diffuse glial infiltration. The perivascular spaces were densely infiltrated by lymphocytes.

In the pons, lesions were characteristically in the region of the floor of the fourth ventricle. There was a diffuse softening; glial infiltration involved the vestibular nuclei, the medial longitudinal fasciculi, and the nuclei of the spinal tracts of the fifth cranial nerves. This process seemed to extend into the fourth ventricle so that in 4 of the 5 cases there was loss of the ependymal lining (Fig. 5) and in one case there was an inflammatory infiltration within the ventricle itself.

In the midbrain there were small glial foci in the midline and around the aqueduct of Sylvius. Glial infiltrates were also noted in the thalamus, putamen and caudate nuclei. Small scattered glial foci were seen in the temporal and parietal cortex in 2 monkeys. The hippocampus, occipital cortex, amygdaloid and hypothalamus were free of lesions.

Serologic Examination

Pre- and post-inoculation serums were available from 2 monkeys for antibody studies. Both animals had neutralizing antibodies in the pre- and post-inoculation serums at a dilution of 1:256, as determined in the tissue culture neutralization test with one of the Virus-B isolates.

Discussion

In studies by Sabin[2] and others[5,10–14] it has been found that many apparently healthy monkeys have neutralizing antibodies to Virus-B. The antibody levels have varied greatly. Sabin and Hurst[2,10] found levels varying from 1:20 to 1:500. Keeble, Christofinis and Wood[5] tested the serums of 100 rhesus monkeys aged 1 to 2½ years and found that 17 had antibodies equal to or greater than 1:8 when tested against a recent Virus-B isolate. In addition, isolation of this virus from normal monkey kidney cell cultures has occurred on several occasions.[12,14] Thus

it would appear that this virus can remain latent in monkey tissues for long periods of time.

Recently, Keeble and co-workers[5] reported on natural Virus-B infection in rhesus monkeys. These animals had vesicular lip lesions and ulcers of the tongue which healed within 7 days. In 5 of 17 animals Virus-B was isolated from the lesions. The central nervous system tissues of 12 of these 17 were examined histologically, and lesions were found in 10. These, as stated previously, consisted of localized involvement of the trigeminal and facial nerve roots and the solitary tract. Similar central nervous system alterations have been seen in our laboratory previously and have been incorporated in the tabulation of lesions (Category 1) found in the course of the safety testing of monkeys up to 1955.[6] Since virus isolation was not attempted prior to 1955, a specific viral diagnosis cannot be made. Recent attempts to demonstrate intranuclear inclusions in this tissue have been unsuccessful.

The lesions we have described were found in cortisone-treated animals after intraspinal, intrathalamic and intramuscular inoculation of inactivated poliomyelitis vaccine. It would appear that the use of cortisone and the intraspinal inoculation trauma caused the reactivation of a latent Virus-B and the development of the severe lesions that we have described. In support of this is the presence of neutralizing antibodies to Virus-B in the serums of two monkeys which were obtained before and after the inoculation of vaccine.

It is of interest that the lesions described in this report are similar to those found in two fatal human cases.[15,16] The reaction of humans who are markedly susceptible to this infection thus seems to be similar to that in cortisone-treated traumatized monkeys which are naturally somewhat resistant.

Since Virus-B infection is a serious and often fatal disease in man,[2,15–18] considerable caution should be used when handling potentially infected tissues. Virus isolation procedures are required on the central nervous system tissues of safety test monkeys showing histologic lesions.[19] Therefore, if the possibility of Virus-B infection is suggested histologically, laboratory personnel can be advised accordingly.

Summary

Lesions of Virus-B infection of the central nervous system of monkeys used in safety tests for poliomyelitis vaccine have been described. These lesions, in contrast to the lesions reported in natural Virus-B infection, are severe and extensive throughout the central nervous system. It seems apparent that these lesions are related to reactivation of latent Virus-B by the intraspinal inoculation of vaccine and by the use of

cortisone. It is important to recognize these characteristic lesions so that virologists can be warned of the dangers of handling infected tissues.

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LEGENDS FOR FIGURES

Illustrations were prepared from tissues stained with gallocyanin.

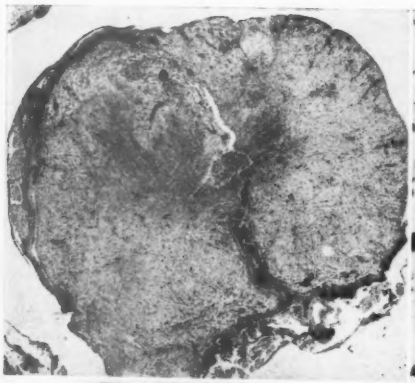
FIG. 1. Lumbar spinal cord of a monkey inoculated intraspinally with poliomyelitis vaccine. There is loss of architecture and necrosis of the entire section. Virus-B was recovered from this tissue. × 9.

FIG. 2. Lumbar spinal cord of a monkey inoculated intraspinally with poliomyelitis vaccine. This is a higher magnification of an area in Figure 1. The extensive necrosis is clearly seen. × 285.

FIG. 3. Nerve roots surrounding the lumbar spinal cord illustrated in Figure 1. There is infiltration by neutrophils, necrosis and demyelinization. × 105.

FIG. 4. Cervical spinal cord of a monkey inoculated with poliomyelitis vaccine. This is the cervical cord of the same monkey shown in Figure 1. There is a small glial infiltration in the anterior horn but no evidence of chromatolysis. × 105.

FIG. 5. Pons of a monkey inoculated with poliomyelitis vaccine. There is loss of the ependymal lining of the fourth ventricle with necrosis and an inflammatory infiltration just below and invading the ventricle. Virus-B was recovered from central nervous system tissue in this monkey. × 105.



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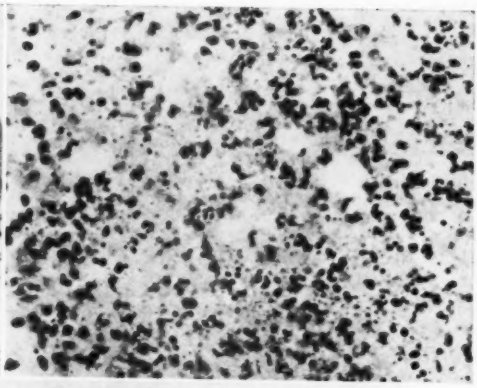

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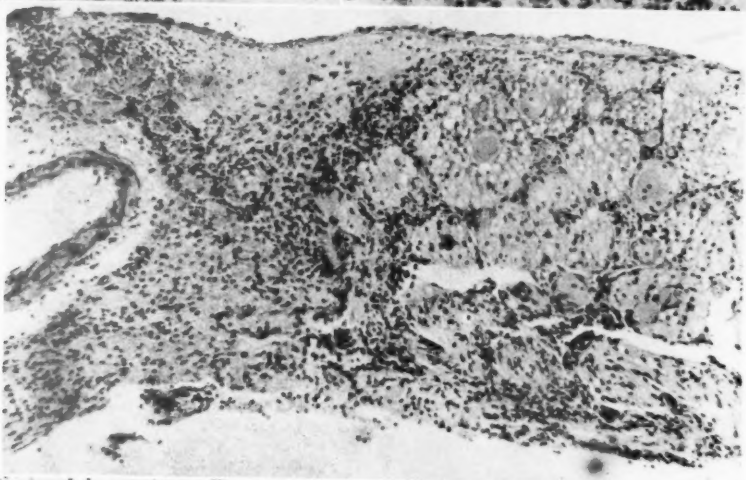

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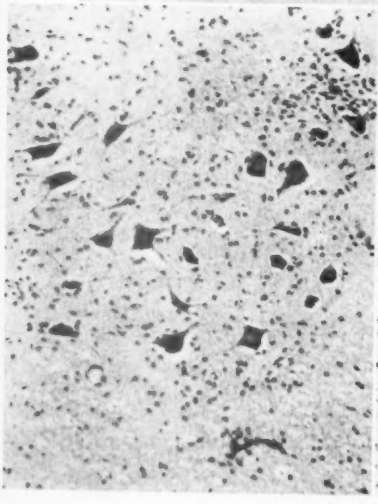

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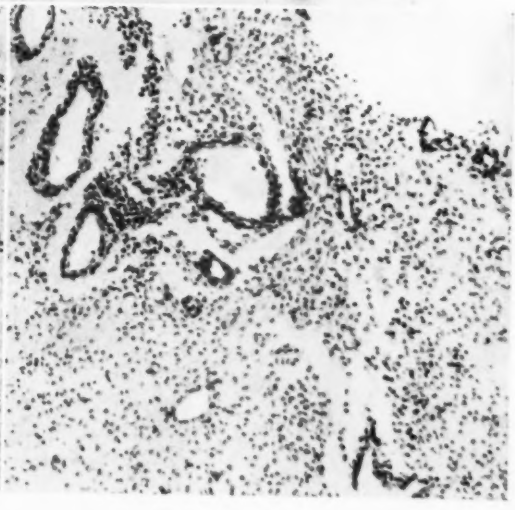
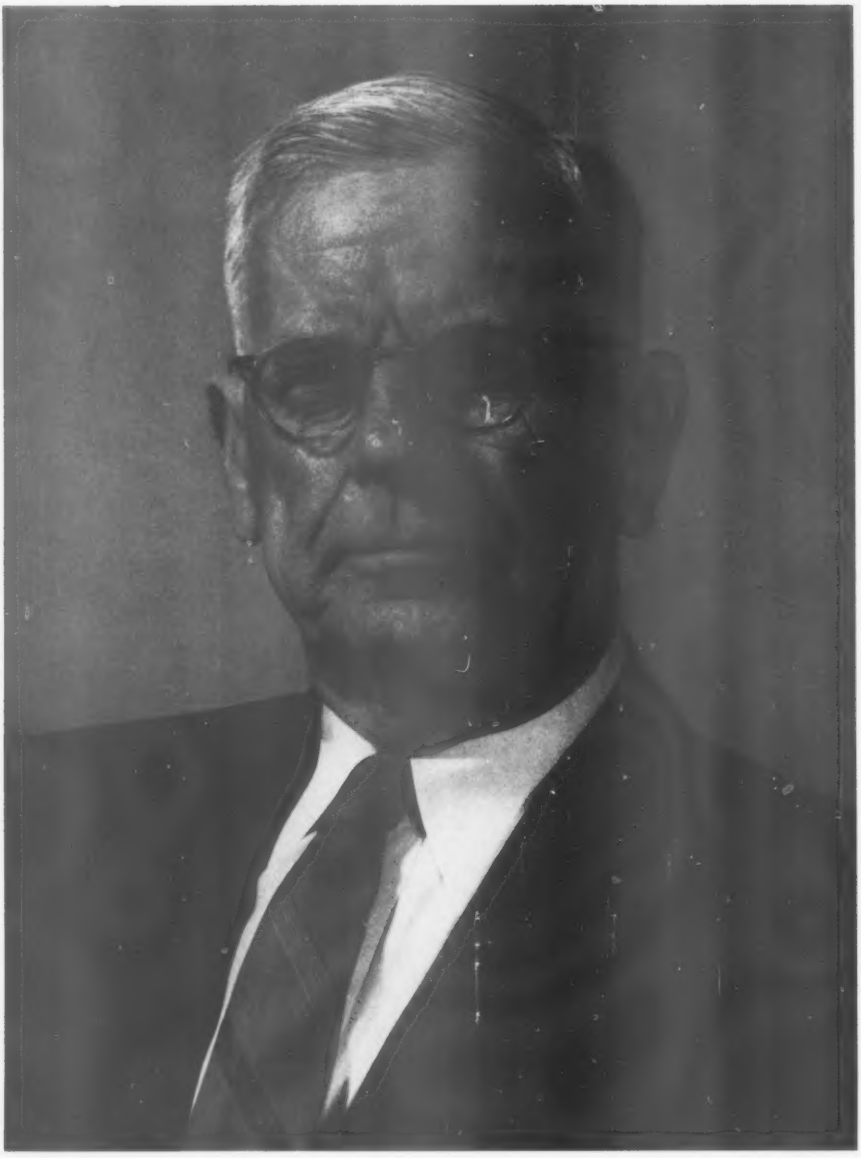


5

ERNEST WILLIAM GOODPASTURE

(1886–1960)



Ernest William Goodpasture

(1886–1960)

ERNEST WILLIAM GOODPASTURE

Ernest William Goodpasture was born October 17, 1886, in Montgomery County, Tennessee, but as a boy and young man he lived in Nashville. Upon receiving his B.A. degree from Vanderbilt University, he entered Johns Hopkins Medical School, graduating in 1912. He remained in the Department of Pathology at Johns Hopkins until 1915 when he joined the faculty of Harvard Medical School. This period, which included two years of wartime service in the Navy, was followed by appointments at the University of the Philippines School of Medicine at Manila and at the William H. Singer Memorial Research Laboratories at Pittsburgh. In 1924, after an absence of 17 years, he was invited to return to Vanderbilt as Professor of Pathology in the reorganized Medical School.

During the years at Hopkins, Harvard, the Philippines and Pittsburgh, Dr. Goodpasture began the investigations which were to bring him world-wide fame, but it was at Vanderbilt that he achieved this renown. For over 30 years there came from his laboratory a series of notable contributions which brought national and international recognition. His best known studies were in the field of infectious disease, especially virus disorders. His most important contribution was the introduction of the chick embryo as an experimental host in the investigation of infection and in the production of vaccines. His experiments were classic examples of simplicity in plan, detailed observation and objectivity. Perhaps the most beautiful were those designed to test the hypothesis of the neural spread of herpes simplex virus. Equally impressive were the investigations demonstrating the infectivity of the inclusions in fowl pox and the determination of the relationship of these inclusions to Borrel bodies.

The eminence of his scientific ability and the importance of his contributions are attested by the numerous honors heaped upon him. Among these were honorary degrees from Yale University, Washington University, the University of Chicago and Tulane University. He was the recipient of the Passano Foundation Award, the Howard Taylor Ricketts Award, the Kober Medal, the Jessie Stevenson Kovalenko Medal of the National Academy of Science, the John Scott Medal, the Award for Achievement in Medical Research by the Southern Medical Association, the John Phillips Memorial Award, and the Gold Headed Cane of the American Association of Pathologists and Bacteriologists. He was elected to the Board of Directors of the International Health Division of the Rockefeller Foundation, to the National Academy of Science, to the American Philosophical Society, and to the Board of Trust, Vander-

bilt University. For many years he was a member of the American Association of Pathologists and Bacteriologists and the American Society of Experimental Pathology, in each of which he served as president. He also served on the Board of Editors, The American Journal of Pathology, and was, for a period, its Editor-in-Chief.

All of these honors and many more were accorded him because of his achievements as a scientist, but he had many other attributes which were even more important. He was a dedicated teacher, and those who were privileged to be his students were fortunate indeed. He was a quiet, modest, thoughtful, kind man, always intent on helping young people. Open rebuke was foreign to his nature, but his soft-spoken suggestions carried great weight. He was a man who encouraged initiative and who delegated authority, a man who backed his junior staff and stood up for his students. All who knew him loved him, admired him and respected him.

Away from his laboratory he was a charming person with many diverse interests, including history, painting, gardening, hunting, fishing, and just plain talking. A wonderful sense of humor and a fine ability to recount stories appropriate to the occasion made him a most perfect companion either in the living room or on a bass stream.

In June 1955, Dr. Goodpasture was retired at Vanderbilt after 30 years of distinguished service to the University, as Professor of Pathology for the entire time and as Dean of the Medical School for 5 years. Following retirement he assumed the position of Scientific Director of the Armed Forces Institute of Pathology where he remained for 4 years before resigning this position to retire and to return to Tennessee. This retirement was short-lived. Friends and former students in the faculty of the Medical School of the University of Mississippi urged him to come to Jackson as Visiting Professor of Pathology. This proved to be a most pleasant interlude, but in the spring of 1960 he again returned to Tennessee where he bought a home and where he set up a laboratory in space provided at Vanderbilt Medical School.

With a life full of service and accomplishment behind him and undiminished physical and mental vigor, Dr. Goodpasture returned to his beloved Middle Tennessee to be near his family and his friends and to engage in those activities which he enjoyed so much. He returned to Vanderbilt Medical School whose fine reputation had resulted so largely from his creative thinking and contributions. In this familiar and congenial environment, in the early evening of September 20, 1960, Ernest William Goodpasture went out to work in his garden. Shortly thereafter this truly great man collapsed and died.

—James R. Dawson, Jr., M.D.